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STUDIES IN MOUSEPOX (INFECTIOUS ECTROMELIA OF MICE)

IV. QUANTITATIVE INVESTIGATIONS ON THE SPREAD OF VIRUS THROUGH THE HOST IN ACTIVELY AND PASSIVELY IMMUNIZED ANIMALS

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(Accepted for publication 3rd September, 1948.)

Burnet's suggestion (Burnet and Boake, 1946) that infectious ectromelia of mice was the murine representative of the mammalian pox viruses has been borne out by subsequent investigations on cross-protection between ectromelia and vaccinia infections in mice (Fenner, 1947a), and by a study of the clinical features of mousepox (Fenner, 1948a and 1948b).

Using the pock-counting technique for virus titration and the haem-agglutinin-inhibition test as a measure of antibody production, it was possible to follow the mechanism of infection in mousepox, and growth curves of the virus in various organs and tissues of infected normal mice were constructed (Fenner, 1948b). In the present paper this method of investigation is extended to mice actively immunized by previous infection with either vaccinia or ectromelia virus, and mice passively immunized with homologous antivaccinia and antiectromelia serum. In addition the 50 p.c. infective doses in normal mice and in actively and passively immunized mice have been determined. The subsequent paper (Fenner and Fenner, 1949) is concerned with the clinical and serological response in mice which had been infected with vaccinia virus before exposure to actively spreading mousepox.

MATERIALS AND METHODS.

The "Moscow" and "Hampstead" strains of ectromelia virus were used, the former as first egg-passage material and the latter after some 50–60 egg passages. "Gillard" vaccinia virus was used for vaccination. Stock virus was prepared from infected chorioallantoic membranes.

The mice were bred in the Hall Institute mouse room and were free from serious enzootic diseases. The stock was heterogenetic and multicoloured.

Virus titrations were carried out by the pock-counting method on the chorioallantois, or by intraperitoneal or pad inoculation of mice. The sensitivity and accuracy of the methods of titration used for constructing the virus growth curves were discussed in detail in a

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TABLE 1.

Vaccinia and ectromelia antihaemagglutinin titres in mice inoculated with vaccinia virus intranasally and subsequently with "Moscow" ectromelia virus in the foot pad. Mice killed five weeks after vaccination.

Controls No ectromelia virus			500 infective particles of ectromelia virus			50 infective particles of ectromelia virus		
E-AHA	V-AHA	E-AHA/V-AHA	E-AHA	V-AHA	E-AHA/V-AHA	E-AHA	V-AHA	E-AHA/V-AHA
40	80	1/2	1,280	1,280	1	160	80	2
80	160	1/2	320	160	2	480	320	3/2
40	80	1/2	640	640	1	640	480	4/3
40	60	2/3	160	160	1	5,280	2,560	2
40	80	1/2	640	320	2	640	640	1
40	40	1/1	160	160	1	320	160	2
20	40	1/2	320	320	1	320	320	1
20	40	1/2	320	320	1	960	640	3/2

previous paper (Fenner, 1948b). In mouse titrations the appearance of a local lesion (of the inoculated foot), post-mortem changes of the liver and spleen, and in doubtful cases the antihaemagglutinin content of the serum were used to establish the fact of infection.

Antibody titrations (ectromelia antihaemagglutinin, E-AHA, and vaccinia antihaemagglutinin, V-AHA) were carried out by estimating the dilution of serum in a 1 p.c. suspension of the erythrocytes of a susceptible fowl which would neutralize four haemagglutinating doses of virus. Blood was usually collected from the axillary vein during ether-induced anaesthesia. The titrations were carried out with 0·25 ml. volumes, the end-point being determined by the pattern of the agglutinated cells at the bottom of the tube. When blood was obtained from the tail veins of previously infected mice, antihaemagglutinin titrations were carried out with 0·05 ml. volumes, as previously described (Fenner, 1947a).

EXPERIMENTAL RESULTS.

The Serological Response of Vaccinated Mice to Subsequent Infection with Ectromelia Virus.

Twenty-four eight-weeks-old male mice were vaccinated by the intranasal instillation of about 10^5 infective particles (estimated on the chorioallantois) of vaccinia virus. The V-AHA titre a fortnight later was not estimated in this experiment, but on numerous other occasions it was found to vary between 160 and 640. The antihaemagglutinin titre against the heterologous ectromelia virus was almost always one half that developed against the homologous vaccinia virus (Fenner, 1947a). A fortnight after vaccination the mice were separated into three groups, one of which was kept as a control group, and in the other two the mice were inoculated in the pad of the foot with either 500 or 50 infective particles (for the mouse) of "Moscow" ectromelia virus. Three weeks later, or five weeks after the original vaccination, all the mice were killed and the V-AHA and E-AHA titres of their sera determined. The results are shown in Table 1.

In the control animals the V-AHA titres had fallen to about one half or one quarter of the usual peak titres, but in all except one case the ratio, E-AHA/V-AHA was 1/2. In both groups of inoculated mice the injected foot was swollen between about the eighth and the eighteenth days. The V-AHA titre either remained stationary at its peak level or rose, and the E-AHA titre rose still further so that the E-AHA/V-AHA ratio became 1 or 2. Subsequent experiments with twenty mice in control and infected groups gave very similar results.

The following serological criteria of infection in vaccinated animals are therefore proposed for mice in which the interval between the inoculation of vaccinia virus and the final titration of the serum is at least five weeks:

(a) E-AHA/V-AHA$\geqslant$1, with E-AHA$\geqslant$160, indicates ectromelia virus infection.

(b) E-AHA/V-AHA$<$1, with E-AHA$<$160, indicates response to vaccinia virus only.

(c) E-AHA/V-AHA$=$1, with E-AHA$\leqslant$80, equivocal result, which suggests ectromelia virus infection of V-AHA a fortnight after vaccination $\leqslant$80.

The application of these criteria in deciding whether certain vaccinated mice had subsequently been infected with ectromelia virus is discussed in the following paper.

The Infective Dose of Ectromelia Virus in Actively and Passively Immunized Mice.

The previous experiment showed that relatively small doses of ectromelia virus caused pronounced local lesions when inoculated in the foot pad of mice which had recently recovered from vaccinia. Experiments were therefore carried out to determine the relationship between the infective doses of two strains of ectromelia virus in normal and vaccinated mice inoculated in the pad of the foot, a method of inoculation which closely resembles natural infection. Subsequently, experiments planned in the same way were carried out with mice which had recently recovered from infection with the relatively avirulent "Hampstead" strain of the virus, and with mice passively immunized with antivaccinia and antiectromelia sera produced in the rabbit.

Eight-weeks-old mice were inoculated intranasally with about 10^5 infective particles of vaccinia virus, and a fortnight later these animals and normal mice of the same age were inoculated in groups of six in the pad of the hind foot with 0·05 ml. of suspensions of ectromelia virus diluted in approximately twofold steps from 10^{-7} to $10^{-9\cdot6}$. The animals were examined for swollen feet every second day from the eighth to the eighteenth day. Survivors were killed and examined on the twentieth day, and their sera titrated for E-AHA in normal animals and E-AHA and V-AHA in the vaccinated mice.

With limiting dilutions of the "Moscow" strain of virus, infection of normal mice determined by the appearance of E-AHA was always accompanied by the appearance of a swollen foot which was apparent by the fourteenth day after inoculation, and by enlargement of the draining lower abdominal lymph node. Death, or less severe involvement of the liver and spleen, frequently occurred. Contact infection sometimes occurred in these cages, but was recognized by the time at which death occurred, by the type of post-mortem lesion and by the occurrence of a primary lesion elsewhere than on the inoculated foot. The "Hampstead" strain was much less virulent, and deaths were rare among mice inoculated with this strain. In addition three subclinical cases occurred in which the serological response was the only evidence of infection.

TABLE 2.

ID_{50} of ectromelia virus in normal mice and mice actively immunized with living vaccinia virus.

	"Hampstead" strain	"Moscow" strain
Chorioallantoic titre	$10^{-7\cdot4}$	$10^{-7\cdot3}$
Normal mice	$10^{-8\cdot5}$	$10^{-8\cdot8}$
Vaccinated mice	$10^{-7\cdot85}$	$10^{-8\cdot25}$

As indicated in the previous section, the ratio of E-AHA/V-AHA in vaccinated mice which were inoculated with ectromelia virus suspensions beyond the limiting dilution (for normal mice) was 1/2 or 1/4. Similar ratios were observed in all mice in two other groups, each of which in the normal mice contained one or two infected animals. Taking the serological response previously described as definite evidence of infection with ectromelia virus, i.e. ratio E-AHA/V-AHA $\leqslant 1$ and E-AHA $\leqslant 160$, two cases of infection occurred in the experiment with the "Moscow" strain of virus in which there had been no clinical or post-mortem evidence of infection. In all the other cases with a positive serological response to ectromelia infection there was swelling of the foot, sometimes slight and transient in nature,

and enlargement of the draining lymph node. In the experiment with the "Hampstead" strain of virus there were seven cases among thirty vaccinated mice in which the only evidence of ectromelia infection was the serological response.

Using the method of Reed and Muench (1938) the 50 p.c. infectivity end-points were determined for each of the four series, a positive serological response being accepted as evidence of infection with ectromelia virus. The results, which are set out in Table 2, show firstly, that with both strains of virus, the infective dose in vaccinated mice is about four times as great as in normal mice, and secondly that the mouse is between ten and thirty times as sensitive to ectromelia infection as the chorioallantois (see also Fenner, 1948b).

Mice which have recovered from an attack of mousepox show an accelerated response when inoculated in the foot with large doses of ectromelia virus. The time relations of this allergic response were described in a previous paper (Fenner, 1948b) but no estimate was made of the size of the dose required to produce it. In order to determine this, the experiment upon the infective dose of virus in mice which had already recovered from infection with "Hampstead" ectromelia virus inoculated into the left forefoot fourteen days earlier, was carried out with suspensions of infected mouse liver diluted in normal saline. An accelerated response, consisting of swelling of the foot reading a maximum on the first or second day and subsiding thereafter, was found in all mice inoculated with suspensions containing 10^6 ID_{50} or more of virus. The reaction was most pronounced with the most concentrated doses.

Contrary to the experience with vaccinated mice, however, none of the mice which had recovered from mousepox infection developed swollen feet after the usual incubation periods for the various doses given. The E-AHA titres of the 120 ectromelia-immune mice used in the experiments had been determined before the challenge inoculations with serum obtained from the tail veins. Only in five of the six mice inoculated with the largest dose of virus ("Hampstead" strain; 10^8 ID_{50}) was there any increase in the E-AHA titre. In an attempt to see whether virus multiplication and invasion had occurred two mice in each group inoculated with the "Moscow" strain were killed on the twelfth day and their feet and spleens tested for virus by the intraperitoneal inoculation of suitably diluted suspensions into normal mice. In no case was virus isolated from the spleen, but in either one or both mice tested for each dilution of "Moscow" virus from undiluted to 10^{-5}, virus was isolated from the inoculated foot. The titre was usually very low, about 10^2 infective particles per gm., but on one occasion reached 10^4. Taking the usual criteria of infection, swelling of the foot, enlargement of the draining lymph node, or a rise in the antibody level, mice which had recovered from an attack of mousepox fourteen days earlier were solidly immune to reinfection. The small amounts of virus found in the feet of some mice on the twelfth day might well have been due to preservation of the inoculum rather than multiplication. It was impossible, therefore, to determine the infective dose in such mice.

The infective dose of the "Moscow" strain of virus in mice passively immunized with antivaccinia and antiectromelia sera prepared in rabbits was determined by inoculating groups of 30 gm. mice intraperitoneally with 1·0 ml. of suitable antiserum. Next day they were divided into groups of four mice and inoculated in the left hind pad with dilutions of "Moscow" ectromelia virus ranging in fivefold steps from 10^{-5} to 10^{-10}. With each antiserum, the antihaemagglutinin titre of the sera of mice killed a day after the intraperitoneal inoculation was 40 using the appropriate virus suspension. The results were completely negative, for the infective dose was the same in the control group as in the two groups passively immunized with antivaccinia and antiectromelia rabbit serum.

Summarizing these results, the infective dose of ectromelia virus was the same in animals passively immunized with antivaccinia serum and anti-

ectromelia immune serum as in normal animals, while in animals actively immunized with living vaccinia virus it was four times as high as in normal animals, and mice which had just recovered from mousepox infection were immune to reinfection.

The Multiplication of Ectromelia Virus in Immunized Mice.

The sequence of events after pad inoculation and the growth curves of virus in various organs of normal mice was described in an earlier paper (Fenner, 1948b). The same method of investigation was applied to the multiplication of the virulent "Moscow" strain of the virus in actively and passively immunized animals. The first experiment in which mice immunized with vaccinia virus were used was conducted in parallel with the original experiment on normal mice described in Fenner, 1948b. The other three, using mice which had recovered from mousepox infection and mice passively immunized with antivaccinia and antiectromelia serum, were conducted in parallel. The general technique was to inoculate a group of suitably treated mice in the left hind pad with about 500 infective particles of "Moscow" ectromelia virus and kill two mice at regular (usually daily) intervals. The concentration of virus in the foot, spleen, blood and skin was determined by titration in mice and on the chorioallantois of developing eggs and the E-AHA and V-AHA titres of the sera were also measured.

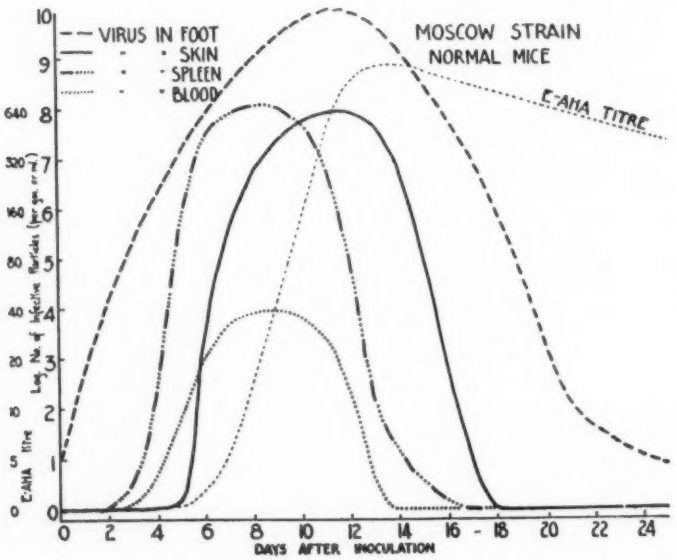

Fig. 1. Growth curves of "Moscow" ectromelia virus in the foot, spleen, blood and skin of normal mice inoculated in the foot with 500 infective particles of virus (modified after Fenner, 1948b).

The mice used for the first experiment had been actively immunized by the intranasal inoculation of 10^5 infective particles of living vaccinia virus five weeks before the inoculation of the ectromelia virus. The second group consisted of mice which had been inoculated in the left forefoot with a dilute suspension of "Hampstead" ectromelia virus four weeks earlier. Pooled sera were obtained from sixty mice which had been inoculated intranasally with dilute vaccinia virus a fortnight before, and from about sixty mice which had recovered from mousepox three weeks or a month earlier. One ml. of serum was inoculated into each mouse, male mice with an average weight of 35 gm. being used. In the group passively immunized with antivaccinia mouse serum tht V-AHA of the pooled serum was 320 and of mice killed 24 hours after passive immunization 40, and the corresponding E-AHA titres in the other group were 320 and 80.

The results of these four experiments are shown in Figs. 2, 3, 4 and 5.

Fig. 1, modified from Fenner (1948b), shows the results obtained with normal mice and the "Moscow" strain of ectromelia virus.

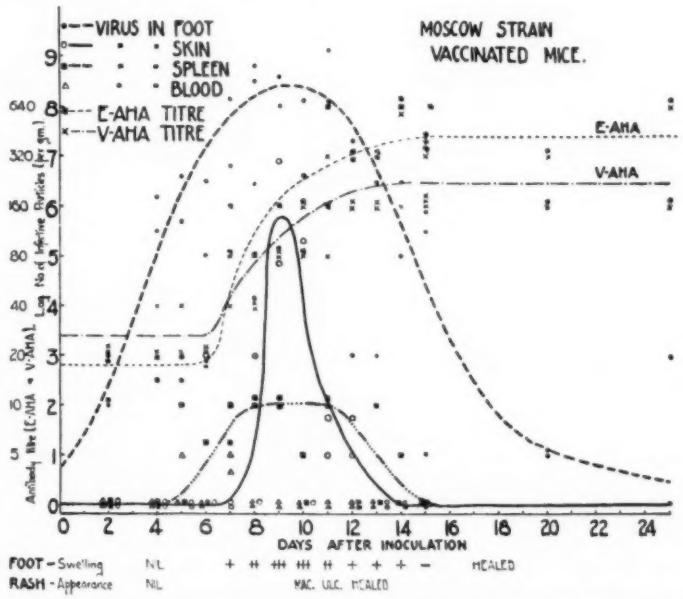

Fig. 2. Growth curves of "Moscow" ectromelia virus in the foot, spleen, blood and skin of mice inoculated in the foot with 500 infective particles of virus five weeks after the intranasal inoculation of 10^5 infective particles of vaccinia virus.

Considering the results as a whole it is apparent that mice recovered from previous infection with mousepox were almost completely protected against subsequent invasion by the parasite, although local multiplication at the site of inoculation sometimes occurred and rarely reached a high titre. Protection in mice actively immunized with living vaccinia virus was less efficient, for invasion of the spleen almost always occurred, but the virus failed to multiply

freely and only in about half the infected animals did infection of the skin occur. Rashes sometimes occurred but were evanescent and mild in character.

Passive immunization with homologous antivaccinia serum, in the doses used in this experiment, was almost without effect upon the progress of infection. The growth curves of virus in the different organs hardly differed from those found in normal mice, and several deaths occurred on the ninth day, when several of the control animals also died.

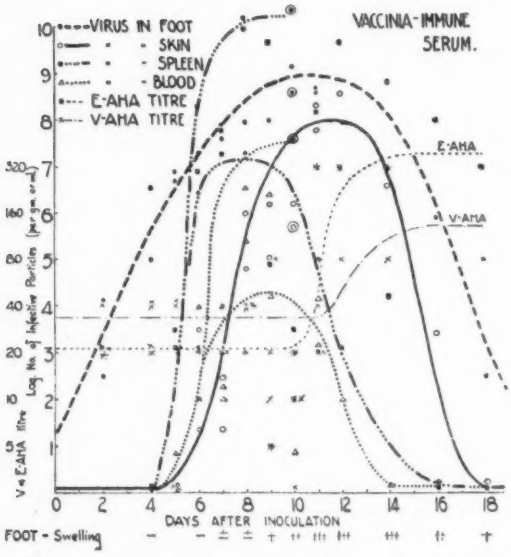

Fig. 3. Growth curves of "Moscow" ectromelia virus in the foot, spleen, blood and skin of mice inoculated in the foot with 500 infective particles of virus twelve hours after the intraperitoneal inoculation of 1 ml. of antivaccinia mouse serum.

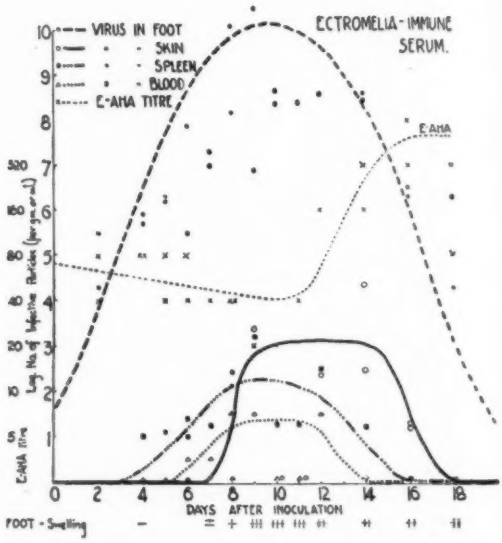

Fig. 4. Growth curves of "Moscow" ectromelia virus in the foot, spleen, blood and skin of mice inoculated in the foot with 500 infective particles of virus twelve hours after the intraperitoneal inoculation of 1 ml. of antiectromelia mouse serum.

The curves obtained in the mice passively immunized with homologous antiectromelia serum closely resembled those obtained in mice actively immunized with vaccinia virus, but virus was more frequently found in the blood and skin.

The results obtained with the different tissues may now be discussed somewhat more fully.

Multiplication of Virus in the Foot.

The curves obtained and the course of the foot lesion in the passively immunized mice did not differ appreciably from those characteristic of normal mice.

Mice recovered from previous mousepox infection gave irregular results. Virus was isolated from the foot in most animals tested, but was often present

only in low concentrations. Visible swelling of the foot was accompanied by a high virus titre. Such swelling appeared earlier, and at lower virus concentrations, than in normal mice, due probably to the hypersensitive state of the animal's tissues.

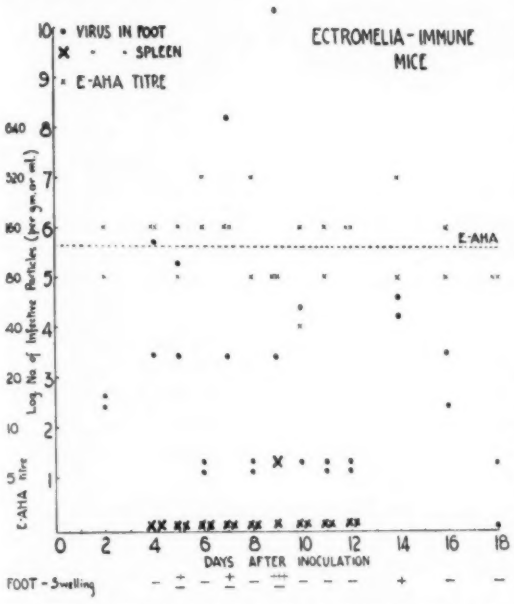

Fig. 5. Titres of virus in the foot and spleen and antibody titres, of mice inoculated in the foot with 500 infective particles of "Moscow" ectromelia virus four weeks after the inoculation of 500 infective particles of "Hampstead" ectromelia virus in the forefoot.

In mice which had recovered from vaccinia infection swelling of the foot appeared to progress slightly more rapidly than in normal mice, and healing certainly occurred more rapidly after the virus titre had reached its peak.

Viraemia and Multiplication of Virus in the Spleen.

The curves for virus in the blood and spleen of mice passively immunized with antivaccinia serum did not differ from those obtained with normal mice. Antiectromelia serum was considerably more effective, and virus never reached high titres, although it was generally present in both spleen and blood between the fourth and the fourteenth days. In mice recovered from previous mousepox infection virus was found in the spleen on one occasion only on the ninth day, in the undiluted suspension of the spleen of a mouse with a very swollen foot which contained 10^{10} infective particles of virus per gm. Active immunization with vaccinia virus was less effective, but although present in the spleen of most animals examined between the fifth and the fourteenth days, it never reached a high titre. Virus was found in the blood of these mice on three occasions only, early in the course of the disease.

Multiplication of Virus in the Skin.

As was expected from the behaviour of virus in the spleen, the growth curve of virus in the skin of mice passively immunized with antivaccinia serum did not differ from normal. With ectromelia-immune serum virus was found in the skin of most animals examined between the eighth and sixteenth days, but the

concentration was never high. Slight rashes were observed in most of the animals.

The occurrence of virus in the skin of mice actively immunized with vaccinia virus was irregular and it sometimes reached a fairly high titre. Rashes, correspondingly, occurred in about half the mice. They were usually slight and healed rapidly. Virus was never found in the skin of the mice which had recovered from a previous infection with mousepox.

The Antibody Curves.

In normal mice the curve constructed through the E-AHA titres at daily intervals began to rise from the base line on the seventh day, after inoculation reached a level of 40 on the ninth day, and reached a peak on the twelfth day.

The antibody titres in the passively immunized mice fell slightly between the first and the tenth day, and then the E-AHA titre rose rapidly to its peak. The period of rapid antibody increase did not differ from that observed in normal animals, except that it may have occurred about a day later in the mice immunized with antiectromelia serum. This may have been related to the much slighter multiplication of the virus in the internal organs of these animals. In the animals passively immunized with antivaccinia serum the V-AHA remained about twice as high as the E-AHA titre until the tenth day, when the latter rapidly increased and the ratio was reversed.

There was no significant rise in the antibody level in the mice which had recovered from mousepox infection before reinoculation. The antibody level of the mouse in which considerable virus multiplication had occurred in the foot, when it was killed on the ninth day was only 80.

In mice actively immunized with vaccinia virus the V-AHA remained about twice as high as the E-AHA until the latter rose on the seventh day and reversal of the V-AHA/E-AHA ratio occurred. In these actively immunized mice the E-AHA titre rose a couple of days earlier than in the normal mice, titres of 80 and 160 being reached on the seventh instead of the tenth day.

Further Experiments on Reinfection of Mice which had Recovered from Mousepox.

The experiments just described showed that mice which had recovered from mousepox a fortnight earlier were solidly immune to reinfection, and after a month's interval virus multiplication occurred only in occasional animals.

In order to see whether the immunity decreased as the interval after the initial infection was lengthened, various doses of virus were inoculated in the feet of mice which had recovered from mousepox twelve months, fourteen weeks, and nine weeks earlier. Seven mice were killed on the sixth day and the virus content of their feet and spleens determined. The foot suspensions were titrated in eggs only, and the spleen suspensions in eggs and mice. The results are shown in Table 3.

TABLE 3.

Virus content of foot and spleen in mice inoculated in the foot with "Moscow" ectromelia virus at various intervals after recovery from mousepox.

Interval between mousepox and inoculation	Dose of virus (ID_{50})	Enlargement of foot		Virus content (ID_{50}/gm.)		E-AHA titre	
		Allergic response	Multiplication response	Foot	Spleen	Before	After
12 months	10^8	—	+	10^9	—	20	—
12 months	10^8	—	+	10^9	0	40	160
12 months	10^6	—	+	10^7	10	10	80
14 weeks	10^8	+	—	$<10^4$*	0	640	1,280
14 weeks	10^8	+	+	$10^{8.5}$	10	320	1,280
14 weeks	10^6	—	+	$10^{8.4}$	10	80	160
14 weeks	10^6	+	—	$<10^4$*	0	160	640

* No virus found at the lowest dilutions tested.

None of the three mice tested twelve months after recovery from mousepox showed an allergic response to the second inoculation of virus, but in all the foot was very swollen on the sixth day and in all the virus content of the foot was high. An allergic response occurred in three of the four younger mice. In one of these, and in the mouse which showed no allergic response the foot became very swollen by the sixth day. As before, the virus content of such swollen feet was high, and that of the feet which appeared normal on the sixth day was low (no virus was detected in the dilutions used). Virus occurred in the spleen in three of the animals in which multiplication had occurred in the foot, but was only detected when the whole of the spleen suspension was inoculated intraperitoneally into one mouse. Multiplication of the virus in the foot can, therefore, occur if the interval between inoculation and infection is longer than two weeks, but invasion of the spleen occurs rarely and multiplication of the virus in the spleen is very limited. The remainder of the mice used in this experiment were killed on the twentieth day, their feet having been carefully observed for the first fortnight. In Fig. 6 the change in antibody content is shown graphically. The mice have been allotted to groups according to the reaction of the foot and the interval between the previous infection and the inoculation, and alongside each curve the dose used for the reinoculation is shown.

Several points emerge from the graph. If we consider first the interval between inoculation and the type of response of the foot, it is apparent when the interval was long there was rarely an allergic response, whereas most of the younger mice inoculated with large doses of virus developed swollen feet twenty-four hours later. The "multiplication response" is the swelling of the foot which occurs some days after inoculation, the exact period depending on dosage. The data in Table 3 show that it is dependent upon multiplication of the virus. Occasionally there was an immediate allergic response, then the foot swelling subsided but recurred several days later. Only two of the old mice failed to show this multiplication response, although it occurred in less than half the younger animals. The usual antibody titre before the inoculation was much lower in the older mice. When there was a multiplication response the antibody titre rose, and when there was none it remained stationary or fell. The only exceptions to this generalization were two old mice inoculated with a large dose of virus in which no enlargement of the foot occurred, but there was a rise in antibody titre; and two of the younger mice in which the initial antibody titre was fairly high and failed to rise, although the feet became swollen several days after inoculation.

Other experiments gave essentially similar results, both in the failure of virus to invade and multiply in the spleen, and the correlation of late enlargement of the foot with virus multiplication in that site and with antibody rise.

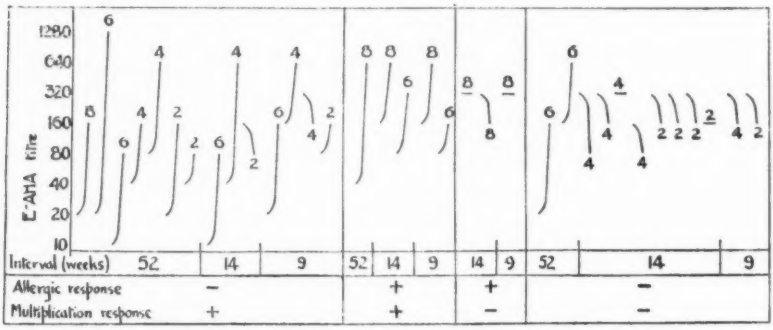

Fig. 6. Changes in ectromelia antihaemagglutinin titres of mice which had recovered from mousepox infection acquired nine weeks to a year previously, when they were inoculated in the foot with various doses of "Moscow" ectromelia virus. The figures shown against each curve are log. number of infective particles of virus inoculated. The interval between first and second antibody titrations was three weeks.

Summarizing these results, it is apparent that previous infection with mousepox confers almost complete immunity to subsequent peripheral inoculation of large doses of virus. The immunity, like the antibody titre, declines with time, but even when the interval between recovery and reinfection is long, the virus usually fails to invade the internal organs, and when it does become established there, fails to multiply freely. The loss of immunity is shown by the increasing frequency with which local multiplication of the virus occurs. There is a close but not absolute correlation between late swelling of the foot due to multiplication of the virus and a rise in the antibody level.

The Response of Mice which have Recovered from Mousepox Infection to Reinfection by the Intranasal, Intraperitoneal and Intracerebral Routes.

All the experiments so far described have been concerned with reinfection by the "physiological" route. It was of some interest to study the response when various doses of virus were inoculated by other routes.

In the course of several experiments in which 50 mice which had recovered from mousepox, acquired at periods varying from two to thirty weeks earlier, were inoculated intraperitoneally with large doses of virus, three animals died within five days of reinoculation with the characteristic lesions of acute mousepox. The pre-inoculation E-AHA titres of these mice were 20, 40 and 80, and their first infections were acquired twenty-six, seven and 4 weeks earlier. This result recalls the observation made in an earlier paper (Fenner, 1947a) that a few mice which had been vaccinated with large doses of vaccinia virus succumbed to acute mousepox within the usual incubation period when challenged a fortnight later by the

intraperitoneal inoculation of large doses of ectromelia virus. In many of the mice which showed no clinical signs of infection, autopsy showed that virus multiplication had occurred on the peritoneal surface of the spleen and other organs. In such mice the antihaemagglutinin titre increased greatly.

The results obtained after intranasal inoculation of the challenge dose were quite different. When an undiluted saline suspension of infected mouse liver was inoculated, death almost invariably occurred in both the control and test mice, but the latter usually died a day or more earlier than the controls. When parallel experiments were carried out with pad and intranasal inoculations it was apparent that such deaths occurred only with dilutions of virus which produced an allergic response on pad inoculation. The inference that these deaths were due to an allergic response in the lungs rather than to multiplication of the virus was borne out by titration of the virus content of the organs of fatal cases amongst normal and ectromelia-immune mice. The results shown in Table 4 were characteristic of several experiments. In the ectromelia-immune mice there was little evidence of virus multiplication in the lungs, which were completely consolidated, and no virus was isolated from the liver or spleen. Amongst the control animals the virus titre of the lungs was higher, consolidation was usually much less widespread, and although the liver and spleen often appeared comparatively normal at autopsy their virus content was always very high.

TABLE 4.

Virus content of organs of mice inoculated intranasally with 10^8 infective particles of "Moscow" ectromelia virus.

Mouse No.	Group	Day of death	Autopsy appearances			Virus titre (ID_{50}/gm.)	
			Lungs	Liver	Spleen	Lungs	Spleen
066	ectromelia-immune	2	4	—	—	2×10^7	0
067		2	4	—	—	2×10^7	0
268		4	2	engorged	engorged	3×10^8	4×10^9
269		4	2	engorged	engorged	2×10^9	6×10^9

When the challenge dose was too small to elicit an allergic response the mice survived and showed no lesions of the lungs at autopsy.

In a few experiments on the intracerebral inoculation of large doses of virus in normal and ectromelia-immune mice the latter died before the control animals, but this subject was not pursued further.

When unphysiological routes of inoculation were used, therefore, the immunity following infection from mousepox sometimes broke down after intraperitoneal inoculation. After intranasal or intracerebral inoculation the immune mice were actually more susceptible than normal animals to doses of virus large enough to elicit an allergic response.

The Infectivity of Mice Actively Immunized with Living Vaccinia Virus and Subsequently Inoculated with Ectromelia Virus.

The experiment on the multiplication of ectromelia virus in mice immunized with living vaccinia virus shows that foot lesions which ulcerated, and skin rashes, occurred on a number

of animals. Titration of the virus content of the spleen and the skin in eight such mice which were killed on the eighth day after the inoculation of the ectromelia virus showed that virus was present in the spleen of every mouse and in the skin of five animals. It is apparent that such animals might be able to transmit the disease.

The experiments to be reported in the following paper, which involve the spread of mousepox through herds of mice actively immunized with vaccinia virus, made it desirable to test the infectivity of vaccinated animals infected with mousepox. An experiment was, therefore, carried out on the same lines as that described with infected normal mice in an earlier paper (Fenner, 1947b), to establish the duration and the degree of risk associated with the exposure of normal mice to such infected, vaccinated animals. A fortnight after the intranasal inoculation of vaccinia virus eight mice were inoculated in the left hind foot with 500 infective particles of "Moscow" ectromelia virus.

Groups of three normal mice were added to the cage containing these infected, vaccinated mice for periods of two days, between the seventh and the twenty-eighth days. The cage was changed at the end of each two-day exposure period and all mice transferred to clean cages. All the vaccinated mice developed ulcers on their inoculated feet but rashes were detected in two only. When normal mice were placed in contact with infected normal animals in the same way, seventeen out of thirty animals contracted mousepox, but in the present experiment only two contact mice out of the thirty exposed were infected, one on the seventh-eighth day exposure and one on the ninth-tenth day exposure. Infected, vaccinated mice can, therefore, infect their normal fellows, but much less freely than do normal, infected animals.

DISCUSSION.

Few investigations have been made on the spread of bacterial or virus infection through the organs of vaccinated animals. The group of Danish investigators led by Oerskov studied the response of actively immunized mice to infection with *Brucella abortus* (Helms, 1932) and to mouse typhoid (Jensen, 1929) and found that in such animals infection was usually restricted to the regional lymph nodes, while in normal animals it became generalized after the first few days with bacteriaemia and considerable multiplication of the organisms in the liver and spleen. No attempt was made in these experiments to determine growth curves of the bacteria in the various organs.

Ipsen (1945) found that when actively immunized mice were inoculated intraperitoneally with a large dose of *Rickettsia mooseri* the growth curves of the rickettsiae in the livers of normal and immunized mice was similar for the first four days, but the curve in the immunized mice then fell away rapidly whilst in the control animals it reached a peak on the fifth and sixth days when most control animals died. A few deaths occurred amongst the vaccinated mice, at the same time (fifth or sixth day) as was usual in normal mice, and the rickettsial concentration in the liver reached the same high titre. This closely resembles the results obtained earlier (Fenner, 1947a) with the intraperitoneal inoculation of large doses of ectromelia virus in mice which had been immunized by previous vaccinial infections. When fatal cases occurred amongst the

vaccinated mice the incubation periods and the post-mortem appearances were identical with those found in control animals.

The present investigation, which is concerned with a natural generalized disease of mice associated with a high degree of immunity in animals which have recovered from previous infection with either ectromelia or vaccinia virus, has given results which resemble in certain particulars both those reported by Ipsen and those found in Jensen's investigations. The growth curves of virus in the local foot lesion in normal mice and mice immunized with vaccinia virus closely resemble Ipsen's data on the rickettsial content of the livers of normal and immunized mice. As in mouse typhoid, active immunity in mousepox greatly decreases the generalization of the disease. Although virus was constantly found in the spleen of mice immunized with vaccinia virus between the fifth and the fourteenth days, multiplication was always slight. Viraemia was only occasionally detected, although the frequent occurrence of virus in the skin indicated that it must have occurred in most animals.

The active immunity which followed recovery from infection with a less virulent strain of ectromelia virus was much more effective than that following vaccinia infection. Generalization of the virus occurred very rarely, and local multiplication in the foot in only a few mice, except when a long interval elapsed between the first and second inoculation of ectromelia virus.

With the doses of serum used passive immunization with antivaccinia serum had no effect on the progress of infection. Comparison of the antibody curves in Figs. 2 and 3 shows that although the immune status of mice, as judged by the antihaemagglutinin content of their serum, was the same, they differed greatly in their response to infection with ectromelia virus.

The protection conferred by passive immunization with homologous anti-ectromelia serum was much more effective, and the curves closely resemble those found in mice which had been actively immunized with vaccinia virus. The efficiency of natural passive immunization in protecting mice from death has been shown in several experiments with suckling mice (Fenner, 1948a, 1949). Preliminary experiments showed that antiectromelia rabbit serum, given intraperitoneally in a dosage of 1·0 ml., which produced an E-AHA titre of 40 in the treated mouse, protected the majority of mice from death and reduced the severity of the disease in the survivors when given even as long as four days after infection with the standard dose of "Moscow" ectromelia virus. The results, shown as the average severity of disease (see Fenner and Fenner, 1949) for the six mice in each group, are given in Table 5.

TABLE 5.

Average severity of mousepox in mice inoculated intraperitoneally with 1·0 ml. rabbit anti-ectromelia serum at intervals after infection with "Moscow" ectromelia virus (500 infective particles).

Interval between infection and inoculation (hours)	0	24	48	96	controls
Average severity of disease	3·5	2·5	1·5	2·8	7·6

Much more extensive experiments were carried out with antivaccinia serum given during the incubation period of mousepox, but as might be anticipated from the results shown in Fig. 3 the results were completely negative. This method of investigation might be used as a model for the study of the mechanism of the prevention of measles by the administration of immune serum during the incubation period. The demonstration by Downie and Dumbell (1947) that antivaccinia and antivariola human sera inactivated variola virus grown on the chorioallantois to an equivalent extent suggests that the treatment of smallpox contacts by vaccination, which is by no means regularly effective (Marsden, 1936), might well be reinforced by the administration of antivaccinia serum or γ globulin.

When a single inoculation of ectromelia virus was made into the foot of mice actively immunized with vaccinia virus, the infective dose of virus was four times greater than that necessary to infect normal animals. Completely inapparent infection, with a positive serological response but no swelling of either the foot or the regional lymph node, was rare with the "Moscow" strain, but with the less virulent "Hampstead" strain of virus several such cases occurred. Passive immunization with antiectromelia and antivaccinia sera had no effect upon the infective dose of ectromelia virus, at least with the amounts and potency of the sera used.

Although in terms of the dosage intervals used in the infection of animals by inoculation the difference in the dose required to infect vaccinated and normal mice is so small, it is apparent that such a difference might be of considerable importance when the disease is spreading naturally. Several of the experiments reported in the following paper bear out this contention.

The establishment of definite serological criteria of infection with mousepox in vaccinated mice provides a method of recognizing those animals, in vaccinated herds exposed to mousepox, which suffer inapparent infection and those in which infection with mousepox does not occur.

SUMMARY.

The progress of infection with ectromelia virus through the organs of mice actively and passively immunized with both ectromelia and vaccinia has been studied, and growth curves of the virus in the organs of such mice have been constructed.

Previous infection with mousepox conferred almost complete immunity to reinfection by pad inoculation for a few weeks. This gradually subsided but even after a year multiplication of the virus occurred only in the site of inoculation and virus could be found in very low concentrations in the spleens of only an occasional animal.

Passive immunization with antiectromelia serum had no effect upon the multiplication of virus in the inoculated foot, but it greatly limited multiplication in the internal organs, and very greatly reduced the clinical severity of the disease.

Active immunization with vaccinia virus was much less effective than previous infection with mousepox. Virus multiplication followed an almost normal course in the foot, although healing occurred somewhat more rapidly than in normal mice. Virus reached the internal organs of most animals, but multiplication in the spleen was limited and secondary viraemia and a rash occurred in only half the infected animals.

Passive immunization with antivaccinia serum, although resulting in antihaemagglutinin titres closely resembling those found with mice actively immunized with vaccinia virus, had no apparent effect on the course of the disease.

The infective dose of ectromelia virus was not affected by passive immunization with antiectromelia or antivaccinia serum, but active immunization with vaccinia virus increased the infective dose fourfold and a previous attack of mousepox rendered all animals completely refractory to reinfection for a few weeks, and only a proportion could be reinfected many months later.

Mice immunized with living vaccinia virus and subsequently infected with mousepox were able to transmit infection to their normal cage mates, but did so much less readily than did normal infected mice.

Serological criteria were established for determining whether mice actively immunized with vaccinia virus had subsequently contracted mousepox.

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STUDIES IN MOUSEPOX (INFECTIOUS ECTROMELIA OF MICE)

V. CLOSED EPIDEMICS IN HERDS OF NORMAL AND VACCINATED MICE

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The intranasal inoculation of moderately large doses of vaccinia virus usually protects mice when challenged a fortnight later with large doses of ectromelia virus inoculated intraperitoneally, but a few mice die of mousepox within the usual incubation period of that disease (Fenner, 1947). Pad inoculation of vaccinated[2] mice with small doses of ectromelia virus caused swelling of the foot and usually enlargement of the draining lymph node, and sometimes a slight rash occurred. The growth curves of the virus in various organs of such mice were described in the preceding paper (Fenner, 1949a). A characteristic rise in antihaemagglutinin titre and reversal of the E-AHA/V-AHA ratio of the serum occurred in the vaccinated animals.

Epizootics of mousepox in herds of normal mice were described in another paper (Fenner, 1948), the method of investigation being the "closed epidemic" of Greenwood *et al.* (1936). Here this method of experiment is extended to the investigation of the effect of vaccination by the intranasal inoculation of vaccinia virus in various doses on the rate of spread of the more virulent "Moscow" mousepox and the resulting morbidity and mortality of the disease. The effect of prolonging the interval between vaccination and exposure to mousepox infection from two to twenty weeks is also described.

MATERIALS AND METHODS.

The "Moscow" strain of ectromelia virus was used as first egg-passage material, and "Gillard" vaccinia virus was used for vaccination. Stock suspensions were prepared from infected chorioallantoic membranes. Virus titrations were carried out on the chorioallantoic membrane by the pock-counting method.

Serum for vaccinia antihaemagglutinin (V-AHA) titrations on the vaccinated mice was obtained by bleeding them from the tail veins. At the conclusion of each experiment all surviving mice were bled from the heart, and the titre of V-AHA and E-AHA in the serum determined by estimating the dilution of serum in 1 p.c. fowl erythrocyte suspension which

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[2] Throughout this paper "vaccinated" is used to denote mice which have been actively immunized with living vaccinia virus.

would neutralize four haemagglutinating doses of virus. The techniques are described in detail in an earlier paper (Fenner, 1947).

The mice were bred in the Hall Institute mouse room, and were free from serious enzootic diseases.

Closed epidemics were set up in the same way as in previous experiments with normal mice (Fenner, 1948). Individual tins were included in each battery of cages so that there was one tin for every twenty mice in the herd, and the degree of crowding of the mice was kept approximately constant by removing one tin from the battery for each twenty deaths. Cage litter consisted of sawdust, and the cages were changed, cleaned and sterilized every second day, all mice being carefully examined during the change-over. The diet consisted of "Barastoc" dog cubes, and an ample supply of tap water was provided from overhead bottles. The room was heated in the winter and for most experiments the temperature varied between 60° F. and 80° F.

EXPERIMENTAL RESULTS.

The Effect of Vaccination with Different Preparations of Vaccinia Virus on the Spread of "Moscow" Mousepox.

Two forms of epidemic were set up. In the first the mixed groups of normal and vaccinated mice and the inoculated "infector" mice were caged together throughout the experiment. In the second, normal and vaccinated mice were either separated on the tenth day after exposure to the infector mice, or they were maintained as separate herds throughout.

When they were six to eight weeks old, male mice in groups of 50 or 100 were inoculated in various ways. Mice were allotted to the various groups, which are designated by letters, so that each contained the same number of mice of the various ages and colours used.

A = Normal control mice which were inoculated intranasally with 0·05 ml. of normal saline containing 10 p.c. horse serum.

B = Mice inoculated with an undiluted suspension of vaccinia virus which had been killed with 0·3 p.c. formalin. The chorioallantoic titre of the suspension before the virus was killed was 4×10^8 infective particles per ml., and the haemagglutinin titre before and after the addition of formalin was 320.

C = Mice inoculated with a relatively dilute suspension of vaccinia virus, a 10^{-5} dilution of the original suspension. Each dose of 0·05 ml. contained about 200 infective particles (titrated on the chorioallantois). This concentration of virus particles had previously (Fenner, 1947) been found to be the smallest dose which gave a haemagglutinin response and protection against the intraperitoneal inoculation of ectromelia virus in the majority of vaccinated animals.

D = Mice inoculated with a moderately large dose of vaccinia virus, a 10^{-2} dilution of the original suspension. Each dose of 0·05 ml. contained about 10^5 infective particles (titrated on the chorioallantois). This had been found to cause a maximum antihaemagglutinin response and to confer maximum protection against the intraperitoneal inoculation of ectromelia virus, without itself producing any symptoms or signs of pneumonia.

A fortnight after inoculation all mice, including the controls, were bled from the tail veins and the sera were titrated for vaccinia antihaemagglutinin (V-AHA). Three days later, when the punctures on the tail were healed the mice were assembled in one battery of cages and female mice which had been inoculated in the pad of the foot with a large dose of "Moscow" ectromelia virus on the previous day were added to the herd, in the proportion of 15 females to each 100 males. Subsequently all the mice were maintained as a single herd throughout the experiment (in Experiment V, Fig. 1), or on the tenth day were separated

again into the component sub-groups A, B, C, and D, and the surviving female mice were divided equally amongst those sub-groups (in Experiment X, Fig. 2). In one experiment two herds of 100 mice (categories A and D) were maintained separately throughout, 15 infected mice being added to each herd (Experiment III).

In all experiments the mice were examined every second day from the tenth day onwards for primary and secondary skin lesions, and on about the thirty-sixth day all survivors were killed, post-mortem examinations were made, and blood was collected for the titration of V-AHA and E-AHA in the serum.

The results of these experiments are given in Tables 1 and 2 and in Figs. 1, 2 and 3. The Figures show the mortality in each sub-group in terms of the total population and the number of daily deaths.

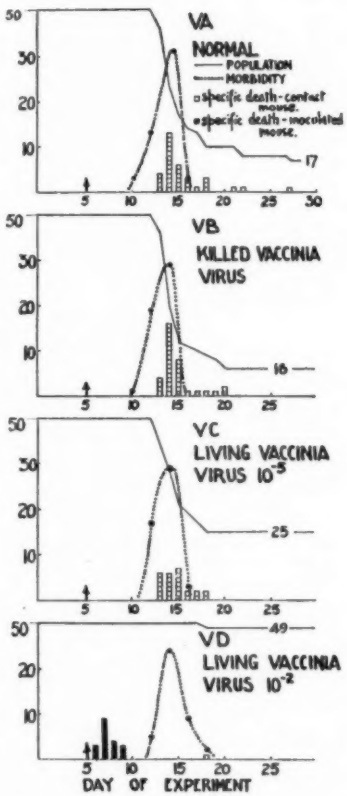

Fig. 1. The mortality and morbidity curves in a herd of mice consisting of vaccinated and normal animals which were exposed to mousepox and maintained as a single herd throughout (Experiment V).

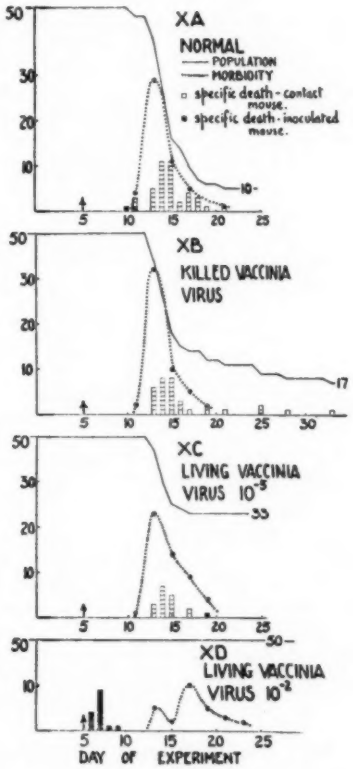

Fig. 2. The mortality and morbidity curves in a herd of mice consisting of vaccinated and normal animals which were separated into the component sub-groups ten days after exposure to the mice infected with mousepox (Experiment X).

The morbidity curves are constructed as described in a previous paper (Fenner, 1948), the number of new cases detected on every second day being shown. New cases were diagnosed by the appearance of a primary lesion, or death without a primary lesion, or by the appearance of the secondary rash if no primary lesion was detected. In the last case the onset of the disease was dated two days earlier than the appearance of the secondary rash. In vaccinated animals, and especially in old vaccinated animals (as in experiment XIII) secondary rashes were often slight, cleared up quickly, and were sometimes hard to distinguish from sores incurred during fighting. In the vaccinated mice in experiment III it is possible that the rash and sometimes the primary lesion was not detected, for the examination of these animals was less careful and thorough than in later experiments. The morbidity curve, and the average severity of the disease in this experiment are, therefore, of doubtful value and have not been included. The severity of the illness in the treated mice has been compared with that of control mice in two ways, firstly by the mortality rate (Table 1), and secondly by a figure called the average severity of disease (Table 2), which has been calculated in the following manner. Each mouse in the herd was allotted a score, consisting of eight for death from mousepox; and in survivors, of one for each of the three degrees of severity of the rash and the lesions of the spleen. A survivor with a severe rash and severe spleen lesions obtained a score of six. The average severity is the figure obtained by dividing the sub-group totals by the number of mice in each sub-group.

TABLE 1.

Mortality rates (expressed as percentages) in each sub-group of experiments involving mice subjected to various vaccination schedules.

Experiments	Groups			
	A Controls	B Killed vaccinia	C 10^{-5} vaccinia	D 10^{-2} vaccinia
III	53	—	—	0
V	66	68	50	2
X	80	66	34	0

TABLE 2.

Average severity of disease including both fatal cases and survivors in each sub-group of experiments involving mice subjected to various vaccination schedules. Explanation of method of scoring in text.

Experiments	Groups			
	A Controls	B Killed vaccinia	C 10^{-5} vaccinia	D 10^{-2} vaccinia
III	5·5	—	—	(0·02)*
V	6·6	6·6	5·5	0·6
X	7·2	6·8	4·1	0·1

* Figure not reliable—see text.

Considering the data as a whole there are two clear-cut groups, A and B on the one hand and D on the other, and one intermediate group C. In experiments V and X the scores for each of the fifty mice were set down in order under the various treatments A, B, C, and D,

ranked first, second, third, and fourth, and a transformation made using Table XX in Fisher and Yates (1943). Analysis of variance of these transformed data showed that the treatment differences were highly significant ($P < 0{\cdot}001$) between all combinations except A and B. There was no significant difference between these two and examination of the Tables and Figures shows that in fact the intranasal administration of a small dose of killed vaccinia virus had no effect whatever. None of the mice in groups A or B showed V-AHA in their serum in the tests made before exposure to mousepox.

TABLE 3.

Vaccinia antihaemagglutinin titres after vaccination.

Group	Vaccinated with	Interval-vaccination to titration	V-AHA titre							
			10	20	40	80	160	320	640	1280
V A	saline	2 weeks	50							
V B	killed vaccinia	2 weeks	50							
V C	vaccinia 10^{-5}	2 weeks	38	2	2	2	3	3	0	0
V D	vaccinia 10^{-2}	2 weeks	0	4	0	1	11	24	8	1
Totals			138	8		53				
X A	saline	2 weeks	50							
X B	killed vaccinia	2 weeks	50							
X C	vaccinia 10^{-5}	2 weeks	30	4	0	9	6	1	0	0
X D	vaccinia 10^{-2}	2 weeks	1	0	1	9	24	15	1	0
Totals			131	5		65				
XIII D	vaccinia 10^{-2}	2 weeks*	0	0	0	2	3	7	0	0
XIII F	vaccinia 10^{-2}	5 weeks†	1	6	11	6	4	0	0	0
		20 weeks	5 | 10	16	16	1	1	0	0	0
			5 | 11							

* Random sample of twelve mice tested. † Random sample of twenty-eight mice tested.

In group D, consisting of mice exposed a fortnight after vaccination with a moderately large dose of living vaccinia virus, there was one death in 250 animals, including the appropriate group from experiment XIII. Such vaccination is, therefore, a very effective protection of mice from death under conditions of severe and continuous exposure. All these mice developed V-AHA after vaccination, the distribution of titres being shown in Table 3. The death occurred in an animal which had the low V-AHA titre of 20 before exposure. This mouse developed a primary lesion on the fourteenth day and died four days later, with an early secondary rash and a very necrotic spleen. The liver showed little gross change.

The severity of the disease in the mice of group D was also greatly reduced as shown by the low values of the "average severity" score and by the rare occurrence of spleen lesions and anything more than slight transient rashes. The frequency histograms in Fig. 3 illustrate these points. In all experiments there were a number of mice in group D that showed no primary lesion or secondary rash during life, and no specific lesions of the spleen or liver at autopsy. In the preceding paper certain serological criteria of ectromelia infection in vaccinated animals were described. These criteria were applied to all surviving animals, with the results shown in Table 4.

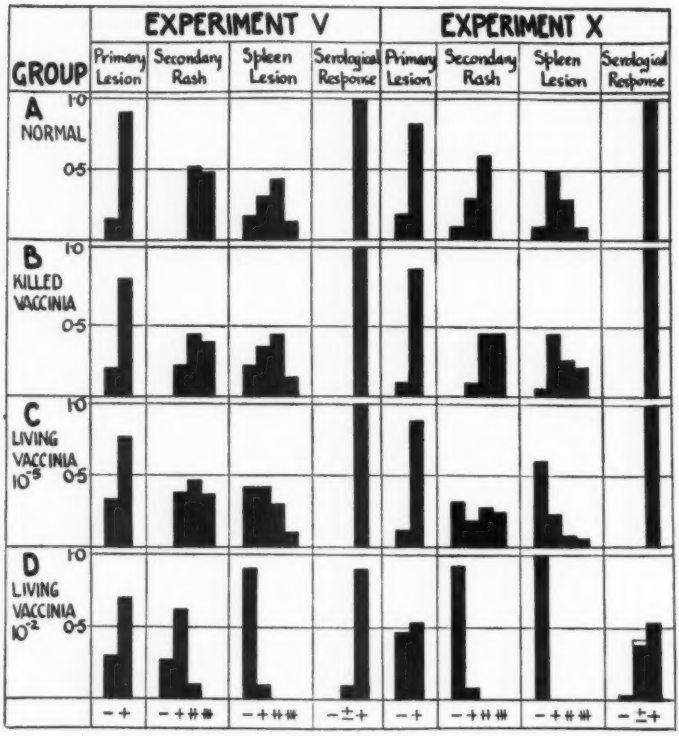

Fig. 3. Frequency histograms of the primary lesions in all mice in Experiments V and X, and of the type of serological response, and the severity of the secondary rash and spleen lesions in survivors in the various sub-groups.

Primary lesions: + = primary lesions seen; — = no primary lesions seen.

Secondary rash: — = no secondary rash seen; + to +++ = slight to severe secondary rash.

Spleen lesions: — = spleen normal at post-mortem examination; + to +++ = slight to severe fibrotic lesions of spleen

Serological response: — = no clinical or serological evidence of mousepox infection; ± = no clinical evidence of infection but serological evidence positive. Unfilled blocks indicate cases in which serological evidence was equivocal; + = clinical and serological evidence of mousepox infection present.

These results confirm the validity of the serological criteria adopted, for out of 159 mice showing clinical evidence of infection, the serological response was positive in all except one animal, in which it was equivocal. Applying them now to the cases in which there was no clinical or post-mortem evidence of mousepox infection, it is apparent that in the majority of cases infection had occurred but had produced no recognized signs. The number of mice which

TABLE 4.

The serological response to ectromelia infection in vaccinated mice.

Group	Evidence of mousepox infection	Number of mice	Serological response*		
			Positive	Negative	Equivocal
III D	Primary lesion only	24	24	0	0
	Lesion of spleen present	2	2	0	0
	No clinical evidence	76	53	19	4
V D	Primary lesion only	8	8	0	0
	Secondary rash present	35	35	0	0
	Lesion of spleen present	3	3	0	0
	No clinical evidence	5	5	0	0
X D	Primary lesion only	23	22	0	1
	Secondary rash present	4	4	0	0
	Lesion of spleen present	0			
	No clinical evidence	23	19	2	2
XIII D	Primary lesion only	18	18	0	0
	Secondary rash present	10	10	0	0
	Lesion of spleen present	2	2	0	0
	No clinical evidence	20	19	0	1
XIII F	Primary lesion only	14	14	0	0
	Secondary rash present	21	21	0	0
	Lesion of spleen present	9	9	0	0
	No clinical evidence	12	12	0	0

* Positive: E-AHA $\geqq$ 160 and E-AHA/V-AHA $\geqq$ 1. Negative: E-AHA $\leqq$ 160 and E-AHA/V-AHA $<$ 1. Equivocal, E-AHA $\leqq$ 80 and E-AHA/V-AHA $=$ 1, probably positive if V-AHA a fortnight after vaccination $\leqq$ 80.

showed no serological evidence of infection varied in different experiments, but in all except III D the proportion was low. Possible reasons for the variability in inapparent infections and failure to acquire infection will be discussed in a later section.

Tables 1 and 2 and Fig. 3 show that in severity of the disease group C was intermediate between groups A and B and group D. More detailed analysis of this group in terms of the V-AHA titre before exposure to mousepox provides an explanation of this, as is shown in Table 5.

TABLE 5.

Mortality rates (expressed as percentages) and average severity of disease in divisions of VC and XC based on V-AHA titre after vaccination.

	Mortality rates		Average severity of disease	
V-AHA titre	$\leqq$10	$\geqq$20	$\leqq$10	$\geqq$20
V C	61 p.c.	17 p.c.	6·3	2·9
X C	57 p.c.	0	6·1	0·9

These figures show that, except for two deaths in mice with V-AHA titres of 20 and 40 in group V C animals, the mice in group C fall into two categories: (a) those which showed an adequate antihaemagglutinin response ($\geqslant 20$) after vaccination, which closely resembled group D in all respects, and (b) those which failed to develop antihaemagglutinin after intranasal inoculation with 10^{-5} dilution of vaccinia virus. These animals behaved in exactly the same way as the normal controls (group A). The antihaemagglutinin, if not the principal antibody concerned in the protection of mice against mousepox infection, is at least a very good index of the presence or absence of the protective antibody.

The Spread of Infection through Normal and Vaccinated Herds.

In a previous paper (Fenner, 1948) the spread of two strains of ectromelia virus through herds of normal mice was compared, and it was concluded that the morbidity curve of the more virulent "Moscow" strain of virus suggested an explosive epidemic, the great majority of mice being infected from the inoculated animals. The height and narrow base of the morbidity curves for the control and unprotected groups of mice in these experiments (VA, VB, XA, XB, XIII A, XIII E) confirms this view. However, the morbidity curves among the vaccinated mice of groups X D, XIII D and XIII F, while accounting for only a proportion of the mice owing to inapparent infections, are much broader than the morbidity curves among normal mice, and even show a tendency to the bimodal form of curve found with the "Hampstead" strain of mousepox spreading through normal mice. In experiment V D, on the other hand, this difference is much less obvious. It was shown in the preceding paper (Fenner, 1949a) that the ID_{50} in vaccinated mice was about four times as great as in normal mice, when the virus was introduced directly into the tissues. This dosage effect probably accounts for the difference between experiments V D and X D, for in the former all the mice were kept in a single herd and the vaccinated mice were, therefore, exposed to the heavy increasing contamination of the environment that occurred when animals in groups A, B and C became infected, while in Experiment X and XIII the vaccinated mice were segregated before this risk developed. This dosage effect is also the probable cause of the small number of inapparent infections in group V D (Table 4), and for the higher average severity of the disease in this group than in X D and XIII D (Tables 2 and 6).

Segregation had no apparent effect on the severity of the disease or its rate of spread through the herd in unprotected mice, for amongst these the infective dose was smaller and there were always many infective animals in the herd, or in the protected mice (V-AHA titre > 20) of sub-groups X C. In the latter case the unprotected mice (V-AHA titre ≤ 10) maintained heavy contamination of the environment with virus.

Discharging primary and secondary lesions in vaccinated mice contain infective virus, as was shown in experiments described in the preceding paper. Such infected animals, therefore, supply the persistent source of infection necessary to explain the bimodal morbidity curves in sub-group D of experiments X and XIII.

The Effect of Prolonging the Interval Between Vaccination and Exposure.

Experiment XIII was carried out in the same manner as Experiment X, but the sub-groups were constituted as follows:

XIII A. Normal 8-weeks-old mice—controls.

XIII E. Normal 28-weeks-old mice—age controls.

XIII D. 8-weeks-old mice which had been vaccinated two weeks earlier with about 10^5 infective particles of vaccinia virus.

XIII F. 28-weeks-old mice which had been vaccinated twenty weeks earlier with about 10^5 infective particles of vaccinia virus.

Thirty female mice infected with "Moscow" ectromelia virus were added to the aggregated herd, which was separated into its sub-groups on the tenth day, an equal proportion of the surviving females being added to each sub-group. All mice were subsequently examined every second day in the usual manner. The mortality and morbidity in these herds is shown in Fig. 4, and the severity of the disease in Fig. 5 and Table 6.

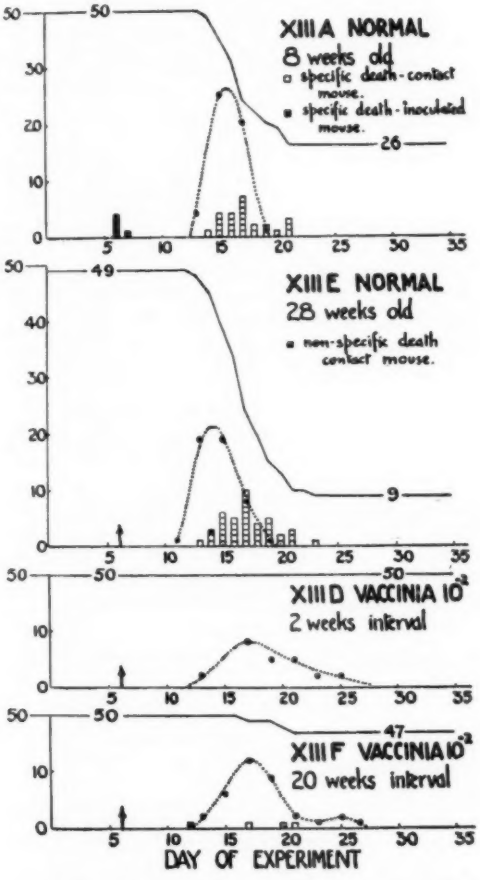

Fig. 4. The mortality and morbidity curves in vaccinated and normal mice at intervals of two and twenty weeks between vaccination and exposure to mousepox. The mice were separated into the component sub-groups ten days after exposure to mice infected with mousepox (Experiment XIII).

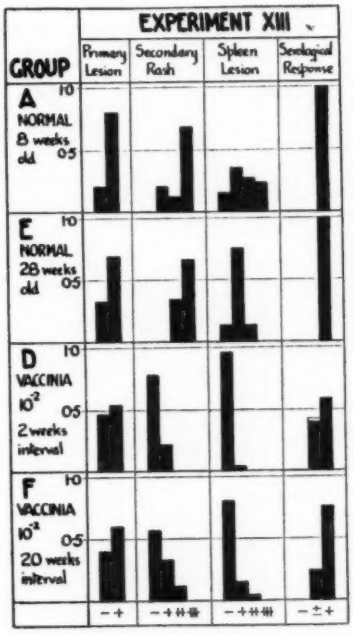

Fig. 5. Frequency histograms of the primary lesions in all mice in Experiment XIII; the type of serological response, and the severity of the secondary rash and spleen lesions in survivors in the various subgroups. (Symbols as in Fig. 3.)

TABLE 6.

Mortality rates (expressed as percentages) and average severity of disease in each sub-group of experiments involving mice exposed to mousepox at various periods after vaccination (Experiment XIII).

	Interval between vaccination and exposure to mousepox			
	Two weeks		Twenty weeks	
	XIII A Controls (8 weeks)	XIII D Vaccinated	XIII E Controls (28 weeks)	XIII F Vaccinated
Mortality rates	48 p.c.	0	81 p.c.	6 p.c.
Average severity	5·9	0·25	7·1	1·2

The disease was much more severe and the mortality rate much higher in the older normal mice than in the young animals. While the mortality rate in the older mice was only slightly greater than was found with eight-weeks-old normal mice in Experiment X, the difference in mortality may be significant. Experiments which have been reported elsewhere (Fenner, 1949b) showed that mice aged forty weeks or more were highly susceptible to the "Hampstead" strain of ectromelia virus, which is of low virulence for eight-weeks-old mice. The higher mortality rate experienced in the herd of older normal mice in this experiment, may, therefore, be due to the lowered resistance which occurs with increasing age. The results in group XIII D agree in all particulars with those obtained in its counterpart, group X D.

Table 3 shows that the V-AHA titre of animals in group XIII F had fallen from the usual level observed two weeks after vaccination until at the onset of the exposure to mousepox there were five animals with titres of 5 or less. Three deaths occurred in this group, all in animals in which the V-AHA was less than 5. Amongst the other mice the disease was slightly more severe than in group D animals, and lesions of the spleen were found at autopsy in 9 animals. There were twelve cases of inapparent infection and fourteen cases in which a primary lesion was the only clinical evidence of infection. Thus while the disease was more severe than among mice exposed only a fortnight after vaccination, protection was still of a high order and greater than would have been anticipated on a basis of the V-AHA titre alone. As pointed out previously (Fenner, 1947) a relatively low titre in an animal which had been adequately vaccinated some time before has not the same significance as when it occurs immediately after vaccination.

The broad-based morbidity curves in groups XIII E and XIII F have been discussed earlier.

DISCUSSION.

The foregoing experiments show that the inoculation of a mouse with a dose of vaccinia virus sufficient to provoke the development of antihaemagglutinin to a titre of 20 or higher often completely protects mice exposed to actively-spreading, highly virulent mousepox. In animals which do show signs of mousepox infection the primary lesion rarely becomes as large or persists as long as it does in normal mice, and the secondary rash is never severe. Fibrosis of the spleen is very rare in adequately vaccinated animals. These observations

are in agreement with those made in the previous paper by the quantitative study of the virus content of organs of vaccinated mice. The degree of protection declines slowly as the period between vaccination and exposure is prolonged.

If a properly vaccinated herd of mice is segregated after exposure to mousepox infection and not mixed with unprotected animals the infection spreads more slowly through the herd, producing a bimodal morbidity curve, and a considerable number of mice suffer inapparent infections or fail altogether to contract the disease. This is probably due to the larger infective dose required in vaccinated mice and the low infectivity of infected vaccinated animals.

There are no comparable experimental data on the spread of a virus infection through a vaccinated herd except the experiments reported by Greenwood and his collaborators (1936) with mice which had been immunized by the intraperitoneal inoculation of killed ectromelia virus followed by a small dose ("attenuated") of living virus. Greenwood found that these mice were almost indifferent (as far as mortality rate was concerned) to the spread of ectromelia through the herd. Some specific deaths were recorded, but there is no indication in Greenwood's monograph that distinctions were drawn between the acute necrosis of the liver and spleen which we regard as unequivocal evidence of the specific nature of the cause of death, and the occurrence of fibrotic lesions of the spleen which merely indicate past infection with ectromelia virus. The advantage in this respect of using vaccinia virus administered intranasally, which causes no changes in the liver and spleen, is obvious. Further, the characteristic change in the E-AHA/V-AHA ratio after infection of a vaccinated mouse with mousepox makes it possible to recognize inapparent infections.

The similarity of mousepox and smallpox has been remarked upon previously, and these experiments may be regarded as a reasonably accurate model of the efficiency of successful vaccination in controlling the epidemic spread of smallpox. The severity and duration of the exposure, especially in the experiment in which vaccinated animals were not segregated, was much greater than is ever likely to occur in human communities infected with smallpox.

SUMMARY.

The intranasal instillation of a dose of living vaccinia virus sufficiently large to produce a reasonable antihaemagglutinin response a fortnight after the infection confers almost complete protection against death upon mice exposed to actively-spreading, highly virulent mousepox. If infection with mousepox does occur there may be no symptoms or signs except an altered serological picture. Clinically apparent cases are always mild, primary lesions small and transient, and secondary rashes of slight severity.

If no antihaemagglutinin response occurs after the intranasal inoculation of vaccinia virus the mouse behaves in just the same way as normal animals when exposed to mousepox infection.

With the passage of time between vaccination and exposure, both the degree of protection and the antihaemagglutinin titre decline, the former to a lesser degree than the antihaemagglutinin titre would suggest.

In a segregated vaccinated herd which has been exposed to mousepox infection the latter disease spreads comparatively slowly and some animals may escape infection completely.

Acknowledgments. We are indebted to Professor F. M. Burnet for advice and criticism and to Mr. E. A. Cornish, Division of Mathematical Statistics, Council for Scientific and Industrial Research, for aid in the statistical treatment of the data.

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STUDIES IN MOUSEPOX (INFECTIOUS ECTROMELIA OF MICE)

VI. A COMPARISON OF THE VIRULENCE AND INFECTIVITY OF THREE STRAINS OF ECTROMELIA VIRUS

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The interaction of the host population and the infecting parasite which determines whether or not an epidemic disease will occur depends upon many factors, some of which are associated with the individual host animals, some with the host population considered as a herd, and some with the infecting micro-organism. At least three properties of the parasite are of importance: its virulence, its infectivity or communicability, and its antigenic character. As the term virulence has been used in so many different senses it is desirable to define it immediately, and in this paper the *virulence* of a strain of ectromelia virus denotes the potentiality of that strain for producing a fatal infection in mice, when spreading naturally or when inoculated by a route which simulates natural infection. As was shown elsewhere (Fenner, 1949), it is also necessary to define the age of the host when discussing the virulence of ectromelia virus, and in all the experiments described here male mice between six to ten weeks old were used. When the potentiality of the strain for producing fatal infections in mice after inoculation by an "unphysiological" route is discussed, the term virulence is qualified by the route of inoculation used.

Infectivity was defined by Greenwood *et al.* (1936) as "the power of a given species of microbial parasite to spread readily from one individual to another among a herd composed of a given species of host, inducing in the host to whom it spreads a significant infection which may take the form either of a fatal or non-fatal illness, or of a latent infection associated with some change in resistance." The term *communicability*, defined by Coburn and Pauli (1941) as "the capacity of the organism to establish itself readily upon the tissues of a new host", may be considered synonymous with infectivity. Both virulence and infectivity probably depend upon a number of different properties of the micro-organism, but the use of these concepts is justified by our ignorance of the component factors.

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The majority of epidemiological investigations made with viruses and bacteria have been concerned with their virulence and their antigenic structure. With a few bacteria, however, the property of communicability or infectivity has been studied, and there is good evidence that it may vary independently of virulence. Greenwood, Hill, Topley and Wilson (1936) obtained clear evidence that with *Pasteurella muriseptica* infections in mice, virulence and infectivity varied independently, for of five strains tested in one experiment two were highly virulent and highly infective, two were highly virulent and of low infective power, and one was relatively avirulent and of low infectivity.

During their investigation of the spread of scarlet fever in an orphanage Keogh *et al.* (1939) found that two type A strains and one type C strain of haemolytic streptococcus spread freely but were not associated with any evident clinical symptoms, while another type A strain spread freely and caused streptococcal sore throat, sometimes accompanied by a rash. White *et al.* (1939) thought that capsulated strains of the haemolytic streptococcus were of greater infectivity than non-capsulated strains.

Coburn (1944) has discussed the problem of the communicability of the haemolytic streptococcus in some detail. He suggested that great infectivity or communicability is a property which certain streptococci may develop when they multiply for some time in the host's tissues, so that when these altered organisms are inhaled by other hosts they multiply readily in the presence of the inhibitory substances in the nasopharynx. Organisms which have not acquired this property of communicability, which Coburn considers to be a rather labile character which may develop in individuals of almost any strain, usually fail to survive in the nasopharynx.

Technical difficulties have in the past precluded all but isolated studies of the infectivity of different strains of animal viruses. Eaton (1940) found that of four strains of influenza virus which were of approximately the same virulence for mice, two which had been through many mouse passages caused contact infection of mice more easily than the other two, which had been passed only a few times in mice.

During experiments with mousepox (infectious ectromelia of mice) spreading through closed communities of susceptible mice, it became apparent that one of the strains of ectromelia virus spread much more rapidly through the herd than the other one, as well as being more virulent (Fenner, 1948a). It was thought at first that this might be due to more massive contamination of the environment by the more virulent virus, but further experiments showed that this was not the only factor concerned. A third strain of ectromelia virus was found to be of high virulence but low infectivity. This paper is concerned with the demonstration that virulence and infectivity may vary independently in viruses as well as bacteria.

MATERIALS AND METHODS.

Virus.

Stock virus suspensions were prepared from infected chorioallantoic membranes which had been inoculated with seed virus three days earlier. At different times four preparations of ectromelia virus were used, and they are designated and defined as follows:

Hampstead (mouse). Dried, infected mouse liver prepared by P. P. Laidlaw in 1935 from mice infected with the strain isolated by Marchal (1930) was passed once in mice in 1946 and again once in 1947. The stock suspension used was first egg-passage material prepared from seed virus consisting of a suspension of mouse liver.

Hampstead (egg). This is the preparation which was used in most previous experiments and was called the "Hampstead" strain in previous papers (Fenner, 1947a, b, c; Fenner, 1948a, b). It is derived from a "Hampstead-mouse" liver preparation which has been passed about sixty times on the chorioallantois during the last twelve years without passage in mice.

Moscow (mouse). Dried mouse liver sent to us in 1946 by Dr. C. H. Andrewes, who obtained it from Professor V. D. Soloviev, was passed twice in mice and stock suspensions consisting of first egg-passage material prepared from seed virus consisting of a suspension of mouse liver.

Moscow (egg). The "Moscow" (mouse) strain was carried through twenty passages on the chorioallantoic membrane before seed virus was prepared from infected membranes.

Virus titrations were made on the chorioallantois by the pock-counting method, or by intraperitoneal or pad inoculation of mice.

Antibody titrations (ectromelia antihaemagglutinin-E-AHA) were carried out by determining the concentration of serum in a 1 p.c. suspension of susceptible fowl cells which would inhibit their agglutination by four agglutinating doses of ectromelia virus.

Mice.

The mice were bred in the Hall Institute mouse room. They are multicoloured and have been outbred for ten years. Male animals six to eight weeks old were used throughout.

Closed epidemics followed the lines laid down by Topley and Greenwood, and the detailed technique used was the same as that described in an earlier paper (Fenner, 1948a).

RESULTS.

The Serological Differentiation of the "Moscow" and "Hampstead" Strains.

Antisera were produced in mice, rabbits, guinea-pigs and rats by single and repeated inoculations of "Moscow" (mouse) and "Hampstead" (egg) preparations of ectromelia virus. Their antihaemagglutinin titre was then determined against four agglutinating doses of each virus. In every case the titre of a particular serum was the same whether tested against the homologous or heterologous strain of the virus. In both rabbits and rats the E-AHA titres of sera from animals subjected to repeated inoculations of virus were about the same as in animals which had been bled after inoculation of a single dose of virus fourteen days earlier.

This result is in agreement with that of Andrewes and Elford (1947) who were unable to detect any antigenic difference between the "Hampstead" and the "Moscow" strains.

The Virulence of Different Strains of Ectromelia Virus in Mice.

The definition of virulence given by Greenwood *et. al.* (1936) referred to the killing power of the micro-organism irrespective of the route of inoculation. It was soon apparent that with ectromelia virus as with most other viruses the route of inoculation had a considerable effect upon the mortality rate and the period of survival before death from mousepox. In order to provide a satisfactory basis for comparison with the mortality rates obtained in epidemics, the site of inoculation used in most experiments was the pad of the hind foot. For comparison, titrations were also carried out by the intraperitoneal inoculation of mice.

The age of the animals used has a considerable effect on the response to infection, very young and very old mice being much more susceptible than mice six to eight weeks old

(Fenner, 1949). All the experiments described below, and the closed epidemics were, therefore, carried out with eight-weeks-old male mice.

Groups of six mice were inoculated in the foot pad or intraperitoneally with 0·05 ml. of various dilutions of the different preparations of virus. Serial tenfold dilutions were used from undiluted to 10^{-7}, and twofold dilutions between 10^{-7} and 10^{-10}. The 50 p.c. end-points for infection (ID_{50}) and for death (LD_{50}) were determined by the method of Reid and Muench (1938), and these are shown in Table 1, together with the titre obtained by titration of the various preparations upon the chorioallantois. The figure given for chorioallantoic titrations is the dilution at which 0·05 ml. would produce one focus on the membrane.

TABLE 1.

The virulence of three strains of ectromelia virus in mice inoculated by different routes or exposed to natural infection.

	Strain of virus		
	"Hampstead" (egg)	"Hampstead" (mouse)	"Moscow" (mouse)
Chorioallantoic titre	$10^{-6 \cdot 4}$	$10^{-6 \cdot 3}$	$10^{-5 \cdot 8}$
ID_{50}, pad inoculation	$10^{-8 \cdot 1}$	$10^{-7 \cdot 7}$	$10^{-7 \cdot 0}$
LD_{50}, pad inoculation	$10^{-1 \cdot 5}$	$10^{-4 \cdot 7}$	$10^{-5 \cdot 1}$
Chorioallantoic titre	$10^{-6 \cdot 0}$	—	$10^{-6 \cdot 8}$
ID_{50}, intraperitoneal inoculation	$10^{-7 \cdot 0}$	—	$10^{-7 \cdot 5}$
LD_{50}, intraperitoneal inoculation	$10^{-6 \cdot 3}$	—	$10^{-7 \cdot 2}$
Closed epidemics			
No. of mice	209	150	347
Mortality rate	11 p.c.	65 p.c.	65 p.c.
Continued epidemics			
No. of mice	134	—	214
Mortality rate	17 p.c.	—	60 p.c.

The specific mortality rates observed in all the closed epidemic experiments in which normal eight-weeks-old mice were used, and in two long-continued epidemics, are also shown, together with the numbers of mice upon which each figure is based.

The virulence is obviously dependent upon the route of inoculation, for the ratio LD_{50}/ID_{50} for "Hampstead" (egg) virus is 5·6 when the virus is inoculated intraperitoneally and $2 \cdot 8 \times 10^6$ when the virus is inoculated in the pad of the foot. The discrepancy is not so great with the "Hampstead" (mouse) and "Moscow" (mouse) strains. Comparison of the mortality rates obtained in natural infections and the LD_{50} after inoculation shows that pad inoculation is a much better index of the expected virulence of the naturally spreading virus than is intraperitoneal inoculation, and this result is not unexpected in view of the mechanism of infection in mousepox (Fenner, 1948b).

Considering now the virulence of the three strains when spreading naturally, or inoculated by a route corresponding to the natural portal of entry, the three strains can be arranged in order of virulence; "Moscow" (mouse) slightly more than "Hampstead" (mouse) much more than "Hampstead" (egg).

The "Moscow" (egg) strain was compared with these three strains by inoculating groups of 24 mice with 0·05 ml. of 10^{-6} dilutions of the four strains of virus. The results of this experiment, arranged in rankits (Ipsen and Jerne, 1944) are shown in Fig. 1. It is

apparent that twenty egg-passages have had no effect upon the virulence of the "Moscow" strain of virus, for in this experiment there was no significant difference between the death rates or survival times of animals inoculated with "Hampstead" (mouse), "Moscow" (mouse) and "Moscow" (egg) strains of the virus.

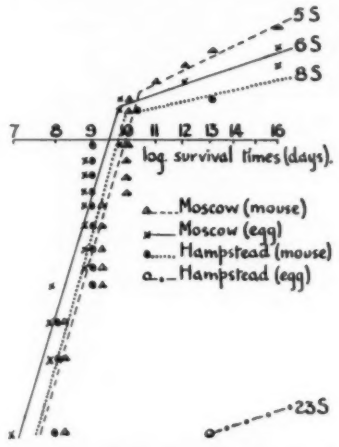

Fig. 1. The survival times of groups of twenty-four mice inoculated with dilute suspensions of four strains of ectromelia virus, plotted as rankits against log survival time (in days). The number of survivors in each group is shown at the right-hand ends of the curves.

The "Hampstead" egg strain is of such low virulence that only one death, occurring on the thirteenth day, was recorded. Deaths occurring on the tenth day or earlier were characterized by very extensive necrosis of the liver and spleen, while in later deaths the lesions of the internal organs were usually healing, the mice were thin and wasted and there was usually an extensive unhealed rash.

The virus titre of the necrotic liver or spleen of acutely fatal cases of mousepox is always in the region of 10^9 to 10^{10} mouse-infective particles per gm. It was thought that the difference in virulence between the "Moscow" (mouse) and the "Hampstead" (egg) strains might be due to their different rates of multiplication in the liver and spleen and one series of experiments (Fenner, 1948b) supported this suggestion. More extensive and detailed experiments upon the multiplication rate of the "Moscow" (mouse), "Hampstead" (mouse) and "Hampstead" (egg) strains of virus in the liver and spleen of eight-weeks-old male mice were therefore carried out. Egg-membrane suspensions of the three strains were prepared and titrated in eggs, and doses of about 500 mouse-infective particles were inoculated in the left hind pad of groups of 72 mice. From the second until the sixth day inclusive four mice from each group were killed and the spleens separately ground and suspended in horse-serum saline. Each suspension was inoculated intraperitoneally into two mice, each of which received one-half of the suspension. This enabled virus to be detected even if present in very small quantities. In Table 2 the number of mice in each group with infected spleens is shown.

TABLE 2.

Invasion of spleen by different strains of ectromelia virus. Number of mice out of four tested daily showing virus in the spleen after the inoculation of about 500 infective particles of each strain of virus in the pad of the hind foot.

Day	Strain of virus		
	"Moscow" (mouse)	"Hampstead" (mouse)	"Hampstead" (egg)
2	0	0	0
3	2	2	0
4	4	4	0
5	4	4	3
6	4	4	4

It is apparent that with the "Hampstead" (egg) strain of virus the delay between infection and entry of virus into the internal organs is much longer than with the other more virulent strains, between which no difference was apparent.

To determine whether the multiplication rates of the different strains of virus differed after they had entered the internal organs, the livers and spleens of six mice in each group were ground and 20 p.c. suspensions prepared on each day from the third to the eighth day after infection. Three sets of suspensions of each organ were prepared, each including organs from two of the mice. These suspensions were titrated in mice and on the chorioallantois as described in a previous paper (Fenner, 1948b). The results were expressed as the logarithm of numbers of infective particles (for the mouse) per gm. of organ. Regression coefficients were determined for the period of logarithmic multiplication and generation times of the different strains of virus in the liver and spleen were also calculated (Table 3).

TABLE 3.

Regression coefficients of virus titre on time and generation times of three strains of ectromelia virus in the liver and spleen.

Strain of virus Period of logarithmic multiplication	"Moscow" (mouse) 4th–7th day incl.		"Hampstead" (mouse) 5th–7th day incl.		"Hampstead" (egg) 5th–8th day incl.	
	Spleen	Liver	Spleen	Liver	Spleen	Liver
Regression coefficients	2·28	1·92	2·52	1·85	2·06	1·58
Generation times (minutes)	190	220	170	260	210	270

The results are in general agreement with those obtained earlier (Fenner, 1948b) but the differences in the multiplication rates of the "Hampstead" (egg) and "Moscow" (mouse) strains of virus are smaller than were then found. The experimental error in the technique is, in any case, rather large, and it seems unlikely that an accurate determination of multiplication rates will be possible until one-step-growth techniques are more fully exploited with the animal viruses.

Comparing the three strains of virus it is evident that the "Moscow" (mouse) and "Hampstead" (mouse) strains resemble each other closely in virulence, in the interval between infection of the foot and entry of virus into the spleen, and in the growth rates of the virus in the liver and spleen. The "Hampstead" (egg) strain differs from them in all these characters, and it appears likely that the lower virulence and the longer interval between peripheral infection and the appearance of virus in the spleen are both expressions of the less rapid multiplication of this strain of virus, in each of the intermediate regions between the periphery and the internal organs, and in the internal organs themselves. This may give the host enough time to commence effective antibody production. Some support for this view is provided by the much higher virulence of the "Hampstead" (egg) strain when inoculations are

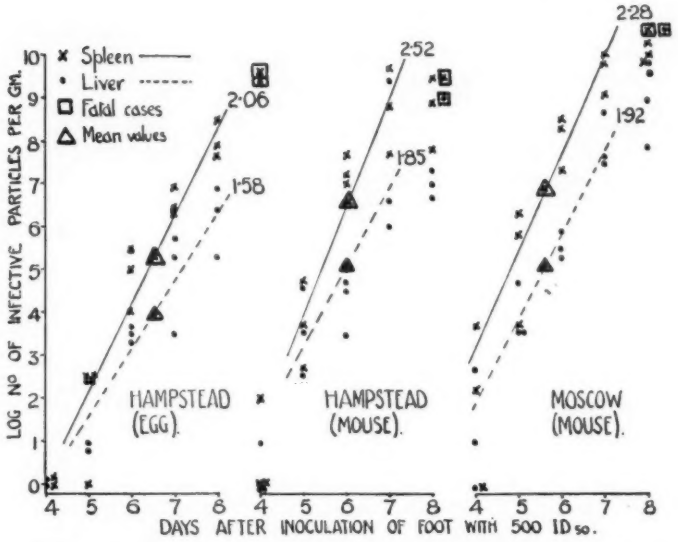

Fig. 2. Growth curves of three strains of ectromelia virus in the liver and spleen of the mouse. The curves are fitted from the mean values and regression coefficients calculated for the period of logarithmic multiplication in each case.

made intraperitoneally so that virus can enter the internal organs directly (see Table 1). Comparison of the survival times of fatal cases inoculated intraperitoneally with equivalent doses of the three strains of virus shows that mice inoculated with the more virulent strains always died a day or more earlier than mice inoculated with the "Hampstead" (egg) strain, the survival periods being much shorter than after pad inoculation of the same dose. Once again, therefore, the "Hampstead" (egg) strain appears to multiply less rapidly, but the direct introduction of the virus into the liver and spleen instead of the delay imposed by the usual passage from the skin, allows the virus to reach a concentration in the liver and spleen of 10^9–10^{10} infective particles per gm. with consequent massive necrosis of those organs and the death of the mouse.

The Infectivity of Different Strains of Virus.

Closed epidemic experiments with the "Moscow" (mouse) and "Hampstead" (egg) strains of virus showed that the former was more highly infective as well as more virulent (Fenner, 1948a). It was thought possible that the apparent greater infectivity of the "Moscow" virus was due to greater contamination of the environment due to the more numerous severe primary and secondary skin lesions which it produced. An attempt was, therefore, made to standardize the severity and time of exposure by selecting, from a large

number of mice which had been inoculated in the foot with various doses of three different strains of ectromelia virus, several pairs of mice in which the severity and stage of development of the primary lesion and secondary rash were the same. Each pair was then placed in a clean cage with six normal mice. After twenty-four hours the contact mice were transferred to clean tins and they were regularly examined for signs of infection. On the thirteenth day after exposure the survivors were killed and their sera tested for E-AHA. The results are shown in Table 4, and it is apparent that while the majority of the mice exposed to the "Moscow" strain of virus contracted the disease only a small and approximately equal number were infected by contact with mice infected with the two varieties of the "Hampstead" strain.

TABLE 4.

Number of infected mice per tin after 24 hours' contact with two mice which had been inoculated in the foot pad 11 days earlier with ectromelia virus. All inoculated mice had early secondary rashes. Six contact mice per tin.

	"Moscow" (mouse)	"Hampstead" (mouse)	"Hampstead" (egg)
Cage number 1	5	0	1
2	6	3	2
3	4	0	2
4	6	3	2
Average number of infected mice per tin	5·3	1·5	1·8

A more strictly quantitative comparison of infectivity was then made by spraying 2·0 ml. of undiluted egg-membrane suspensions of the different strains of virus on 40 gm. of sawdust spread evenly over the floors of clean cages. The virus suspensions were titrated on the chorioallantois, and groups of ten mice were inoculated in the pad with small doses to compare the virulence of the preparations used. Six normal mice were then added to each cage, together with adequate food. Twenty-four hours later the mice were transferred to a clean cage and they were regularly examined thereafter for the development of primary lesions. When a mouse developed a primary lesion it was removed from the cage, and all the mice remaining on the fourteenth day were killed and examined by autopsy and serologically for evidence of infection.

In the first two experiments eight cages were included in each series, three strains of virus being tested. In the third experiment four cages were used in each series, and virus was reapplied at the end of twenty-four hours. The mice were removed after forty-eight hours' exposure.

The results are shown in Table 5. They are quite clear-cut in the first two experiments. When approximately the same amount of virus was used to contaminate the cages and the mice were exposed for a short, limited period the majority of those exposed to the "Moscow" strain contracted mouse-pox and only an occasional mouse in cages treated with either of the "Hampstead" strains was infected. The relative virulence of the three strains of virus was the same as was found in earlier experiments (Table 1).

TABLE 5.

Number of infected mice per tin after exposure for a limited time to approximately the same concentration of three different strains of ectromelia virus sprayed on the cage litter. Six mice in each tin.

	Strain of virus		
	"Moscow" (mouse)	"Hampstead" (mouse)	"Hampstead" (egg)
Experiment 1. 24 hours' exposure			
Titre of suspension*	$10^{8 \cdot 5}$	$10^{8 \cdot 4}$	$10^{8 \cdot 3}$
Mortality after pad inoculation†	0/10	3/10	10/10
Cage number 1	5	0	1
2	4	2	1
3	2	1	0
4	4	0	1
5	5	0	0
6	2	0	0
7	1	0	0
8	4	0	1
Average number of infected mice per cage	3·4	0·4	0·5
Experiment 2. 24 hours' exposure			
Titre of suspension	$10^{8 \cdot 0}$	$10^{7 \cdot 9}$	$10^{7 \cdot 6}$
Mortality after pad inoculation	1/10	1/10	9/10
Cage number 1	3	0	1
2	2	0	0
3	2	2	0
4	3	0	2
5	5	0	1
6	5	1	0
7	3	0	0
8	4	0	1
Average number of infected mice per cage	3·4	0·4	0·6
Experiment 3. 48 hours' exposure Two applications of virus			
Titre of suspension	$10^{9 \cdot 2}$	$10^{8 \cdot 5}$	$10^{8 \cdot 8}$
Cage number 1	6	4	2
2	5	6	5
3	6	3	3
4	6	3	4
Average number of infected mice per cage	5·8	4·0	3·5

* Titre determined by chorioallantoic titration and expressed as infective (focus-producing) particles per ml.

† Numerator = survivors, denominator = inoculated mice. 0·05 ml. of 10^{-6} dilution inoculated in foot pad.

Doubling the time and severity of the exposure (Experiment 3) increased the number of infected mice in the "Moscow" cages to almost 100 p.c., and there were considerable increases in the other cages also, so that analysis of variance of the results in this experiment failed to reveal significant differences between the treatments. This provides an explanation for the results obtained when closed epidemics were set up with the three strains of virus,

with the results shown in Fig. 3. The experiments with "Moscow" (mouse) and "Hampstead" (egg) virus were described in an earlier paper (Fenner, 1948a), but that with the "Hampstead" (mouse) strain has not been reported.

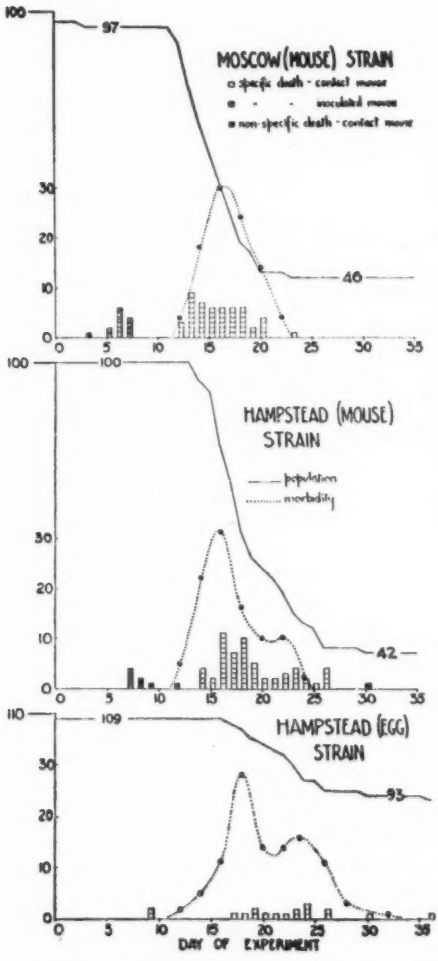

Fig 3. Morbidity and mortality curves for closed epidemics with three strains of ectromelia virus. Each epidemic was initiated by adding 15 mice inoculated on day 0 with the appropriate strain of virus to 100 normal eight-weeks-old male mice.

There was no significant difference between the mortality rates in the "Moscow" (mouse) and "Hampstead" (mouse) epidemics, but the mortality rate in the "Hampstead" (egg) experiment was much lower than in the other two. The unimodal narrow-based morbidity curve which occurred in the "Moscow" (mouse) experiment indicates that the infectivity of the virus was high, and the majority of cases were probably infected from the inoculated mice. In the "Hampstead" (egg) epidemic there were two peaks on the morbidity curve, and consideration of the length of the incubation period indicates that the second peak corresponds to secondary cases. The infectivity of the "Hampstead" (egg) strain of virus spreading naturally was thus much less than that of the "Moscow" (mouse) strain. The morbidity curve of the "Hampstead" (mouse) epidemic was intermediate in type. It showed a definite low secondary peak, and there was a corresponding secondary peak in fatal cases, which were all due to acute mousepox, but the infectivity did not appear to be as low as in the "Hampstead' (egg) epidemic. The explanation for this is furnished by the experiment in which mice were exposed for forty-eight hours to two applications of virus to the cage litter. It can be seen that under these conditions of severe exposure the infectivity of both the "Hampstead" (egg) and "Hampstead" (mouse) strains approached more closely to that of the "Moscow" (mouse) strain. The much greater severity of skin lesion (primary and

secondary) in mice infected with the "Hampstead" (mouse) strain of virus caused more extensive contamination of the environment than occurred in the "Hampstead" (egg) strain epidemic, with a corresponding decrease in the average period before infection and therefore an apparently greater infectivity of the "Hampstead" (mouse) strain of virus. The results of these closed epidemics are therefore compatible with the view that the three strains of virus had different properties, viz.: "Moscow" (mouse), high virulence and high infectivity; "Hampstead" (mouse), high virulence and low infectivity; "Hampstead" (egg), low virulence and low infectivity.

DISCUSSION.

As Dubos (1945) has pointed out, the virulence of bacteria is a very complex property, the summation of many complementary attributes. The situation with which we have been concerned is probably simpler, for the virus particle is chemically much simpler than the bacterial cell, and passage is invariably made in living animals and the dissociation encountered in the passage of bacteria on lifeless media does not occur.

Further, the strains of ectromelia virus which have been studied in this paper resemble each other closely in the complex mechanism of infection and production of lesions, differing only in the rate at which the lesions are produced and their severity. In virus diseases visible lesions occur only when the virus titre of the affected organ has almost reached its maximum level (Rivers, 1939) and this has been shown to be true with both the "Moscow" (mouse) and "Hampstead" (egg) strains of ectromelia virus (Fenner, 1948b). If the virus titre fails to reach a level of about 10^9 infective particles per gm. of liver, the mouse usually survives the acute disease. The evidence given in this paper suggests that in natural infections or after pad inoculation the "Moscow" (mouse) and "Hampstead" (mouse) strains of the virus frequently reach this concentration because at all stages of the complex course of the disease the number of infective particles increases more rapidly than occurs with the "Hampstead" (egg) strain.

The essential difference may lie not in the reproduction rate of the virus *per se*, but in the ease with which virus particles are liberated from the inclusion bodies in which they develop in the skin and usually in the lymph nodes also. In the liver and spleen, where the virus multiplies freely and not within inclusion bodies, this difference would play a less important rôle and consequently the actual multiplication rates might be very similar.

At the risk of too great a simplification of the problem, it appears that the virulent strains are those which multiply rapidly and thus outstrip the defence powers of the host animal, while the strains of low virulence are those in which the slower rate of multiplication of the virus allows antibody production and possibly other defence mechanisms to come into effective play and overcome the infection.

Much less is known of the factors concerned in the complex property of infectivity of bacteria and viruses than is known of the attributes concerned with virulence. However, there is good evidence that in bacteria infectivity may vary independently of virulence, and the experiments described here show that this may occur in viruses also. Apparently increased infectivity may be due to greater liberation of virus into the environment, but when this factor has been eliminated great differences between the infectivity of different strains of virus still occur. If the minimum infecting dose of a highly infective strain were smaller than that of a strain of low infectivity, this might explain the observed difference of infectivity, but the ID_{50} of the three strains of virus when inoculated into the foot appears to be about the same, when suspensions containing equivalent numbers of infective particles (as titrated in another animal—the chorioallantois of the chick embryo) are used. These two factors, lighter contamination of the environment and large infective dose, have been shown to be important in decreasing the apparent infectivity of the "Moscow" (mouse) strain of virus circulating in a population of vaccinated mice (Fenner and Fenner (1949).

The possibility that the difference between the infectivity of the "Moscow" and "Hampstead" strains is due to differences in the growth of the virus in the egg membrane, so that the former is free from some inert material which may surround the latter, is excluded by the experiment shown in Table 4, in which infected mice were the source of the contamination. Considerable differences in the viability of the different strains under the conditions of heat and moisture prevailing in the cages might explain the observed differences in infectivity. While no systematic comparisons have been made, it was shown (Fenner, 1947b) that the "Hampstead" (egg) strain of virus was highly resistant, for it survived for eight weeks when an egg membrane suspension was dried in a Petri dish and left on the laboratory bench.

Thus the factors responsible for the observed differences in infectivity of the three strains of virus for normal mice elude definition. The demonstration of such reproducible differences in a virus as easily handled in the laboratory as is ectromelia virus does, however, provide the material for a detailed investigation of the problem.

Andrewes and Elford (1947) found that their "Hampstead" strain, which had never been passed in eggs (Andrewes, personal communication), was much less infectious than the "Moscow" strain. Their protocols show that it was also somewhat less virulent after pad inoculation, but it was certainly much more virulent than the "Hampstead" (egg) strain of virus. Although no detailed comparison is possible with the available data, the Hampstead strain of virus at present used at the National Institute for Medical Research appears to resemble our "Hampstead" (mouse) strain in both infectivity and virulence.

Little can be said of the manner of production of the relatively avirulent "Hampstead" (egg) strain from the "Hampstead" (mouse) strain. The egg passages were made during 1935–6, again in 1942–3 and again in 1946–7, and for various reasons no attempt was made to repeat the process with the "Hampstead" (mouse) strain. Twenty egg passages had no effect on the virulence of the "Moscow" (mouse) strain of virus.

SUMMARY.

The virulence and infectivity of three strains of ectromelia virus have been studied. The three strains had various combinations of these properties. The "Moscow" (mouse) strain was highly virulent and highly infective, the "Hampstead" (mouse) strain was highly virulent but of low infectivity, and the "Hampstead" (egg) strain, which had been passed 50–60 times in eggs, was of low virulence and low infectivity.

It is suggested that high virulence may be associated with rapid multiplication of the virus in the mouse tissues. No cause could be found for the observed differences in infectivity.

The quantitative methods available for the study of multiplication of the virus and the determination of the infectivity of different strains make ectromelia virus a useful organism for the further study of virulence and infectivity in animal viruses.

Acknowledgment. I am indebted to Professor F. M. Burnet for advice and criticism.

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STUDIES IN MOUSEPOX (INFECTIOUS ECTROMELIA OF MICE)

VII. THE EFFECT OF THE AGE OF THE HOST UPON THE RESPONSE TO INFECTION

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The age-incidence of infectious diseases depends upon a number of factors, the most important being the immunity resulting from previous attacks in the animal or its mother, the difference in the severity of exposure in different age-groups, and the different reactions of host animals of different ages to the same dose of the same new parasite. It is not possible to separate these factors entirely when studying the endemic or epidemic prevalence of natural diseases, but a large amount of epidemiological investigation has shown the importance of the first and second. This paper is concerned principally with the third factor, that is the effect of the age of the host animal upon its reaction to infection with a parasite of which it has had no previous experience. Human medicine abounds with examples of the variation of severity of different diseases in different age-groups. Old people are particularly susceptible to some diseases, notably typhus, psittacosis and St. Louis encephalitis. The very young often suffer severe generalized disease from infection with organisms which remain localized in older individuals, e.g. staphylococcal osteomyelitis, meningococcal, tuberculous and *Haemophilus influenzae* meningitis. On the other hand, some infections are notably mild in the very young, for instance typhus and yellow fever. Certain diseases, such as pandemic influenza of 1918–19, exert their maximum lethal effect upon the young adult group.

Mousepox is a natural virus disease of mice which is associated with an initial local lesion of the skin, but invariably becomes generalized in non-immune animals. The mechanism of infection in mousepox has been studied in some detail (Fenner, 1948b) and methods are available for the accurate study of the natural by spreading disease (Fenner, 1948a). It therefore seemed worthwhile to investigate the effect of age of the host upon the response to infection with mouse-pox.

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MATERIALS AND METHODS.

Stock suspensions of virus were prepared from infected chorioallantoic membranes. The "Hampstead" strain of virus was prepared from seed virus which has been passed 50–60 times in eggs and infected mouse liver was used as seed virus for the "Moscow" strain.

Virus titrations were made on the chorioallantois by the pock-counting method. Antibody titrations (ectromelia antihaemagglutinin-E-AHA) were made by determining the dilution of the serum in a 1 p.c. susceptible fowl red-cell suspension which would inhibit agglutination by four haemagglutinating doses of virus. When ample supplies of serum were available, tests were carried out with standard volumes of 0·25 ml., but when the mouse had to be preserved, blood was obtained from the tail veins and 0·05 ml. used as the standard volume. The latter technique is described in detail in an earlier paper (Fenner, 1947).

The mice used were multicoloured animals from the Hall Institute mouse room. Except for suckling mice and their mothers, male mice only were used. Suckling mice were kept with their mothers until they were three weeks old. The only serious enzootic disease in the stock was a low-grade chronic pneumonia usually associated with a Gram-negative bacillus, which occurred only in mice more than six months old. Mice with this infection could usually be recognized because of loss of weight, rapid respiration, hunched back, and sometimes cyanosis, and they were not used in experiments with ectromelia virus.

Closed epidemics were set up in round tins so arranged that one tin was available for every twenty mice. Cage litter was sawdust and the mice were given an ample diet of "Barastoc" dog cubes and tap water. Cages were changed every second day, all mice being examined at the time of change over.

RESULTS.

The Effect of Age upon the Mortality Rate after Peripheral Inoculation.

The sequence of events after inoculation of a small dose of ectromelia virus in the foot pad closely resembles that following natural infection. Such a method of introduction of a known small dose of virus was therefore used to test the response of suckling mice, eight-weeks-old mice, and fifty-six-weeks-old mice to two strains of ectromelia virus, with the results given in Table 1.

The results are quite clear-cut. With eight-weeks-old mice the usual difference in mortality rates between infections with the two strains of virus (Fenner, 1949) occurred, but in the old mice there was no significant difference in the mortality rates, almost all animals dying of the acute disease. The survival times were about two days longer with the "Hampstead" strain.

The mortality rates were high and the survival times short among the suckling mice, but the "Hampstead" strain was less virulent than the "Moscow" strain, for there were five survivors in the latter group and none in the former and the survival times were about two days longer in the fatal cases of the "Hampstead" group. All survivors had severe attacks of mousepox and showed healing patchy necrosis of both liver and spleen at autopsy. In the fatal cases involvement of both kidneys was common, haemorrhagic foci occurring in half the animals of the "Hampstead" group. Occasionally similar foci were found in the lungs also. The E-AHA titres of the four survivors tested were 160, 160, 80, 80; which are reasonably high titres but are considerably lower than would be expected in adult mice after severe mousepox.

TABLE 1.

Survival times of mice of various ages inoculated in left hind foot with small doses of ectromelia virus.

Strain of virus and dose	Age of mice	Average weight (gm.)	Size of inoculum (ml.)	No. of mice	Survival time (days)									Survivors
					5	6	7	8	9	10	11	12	15	
"Moscow"	10–14 days	7	0·01–0·02	20	2	14	4							0
10^4 ID_{50}	8 weeks	34	0·05	20			4	8	3	1	1		1	2
per ml.	56 weeks	48	0·07	17			2	9	6					0
"Hampstead"	10–14 days	7	0·01–0·02	19			5	5	1		1	1	1	5
4×10^4 ID_{50}	8 weeks	34	0·05	20					1					19
per ml.	56 weeks	48	0·07	16					2	8	5			1

The Effect of Age upon the Mortality Rate and Morbidity Rate in Closed Epidemics.

The age effect was most obvious with the less virulent "Hampstead" strain, and a closed epidemic was established by adding fifteen mice which had been inoculated in the hind foot with a large dose of this strain of virus, to a herd of one hundred male mice which consisted of fifty eight-weeks-old and fifty forty-four-weeks-old mice. On the tenth day, the two age groups were separated and half the surviving inoculated mice added to each herd. Thereafter they were maintained separately and examined every second day, and the experiment was concluded on the thirty-fifth day by killing the survivors and determining the E-AHA titre of their sera. The results are shown in Fig. 1.

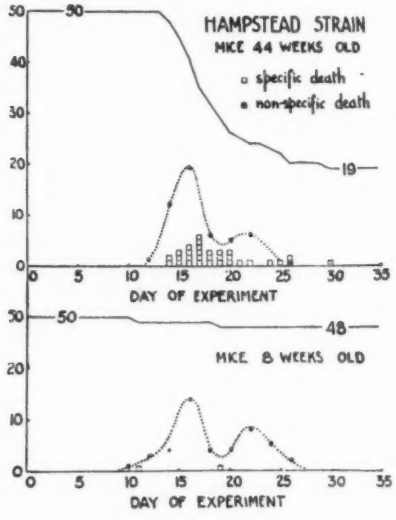

Fig. 1.

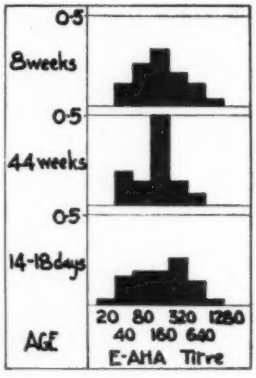

Fig. 2.

Fig. 1. Mortality and morbidity curves in herds of mice of different ages exposed to infection with the "Hampstead" strain of ectromelia virus.

Fig. 2. Frequency histograms showing the E-AHA titres in mice of different ages (a) the survivors of naturally acquired "Hampstead" mousepox, eight weeks' old at time of first exposure, (b) survivors of naturally acquired "Hampstead" mousepox forty-four weeks' old at time of first exposure, (c) the survivors of natural infection acquired as suckling mice partly protected by passively-received antibody.

The epidemic amongst eight-weeks-old mice followed the usual lines for the "Hampstead" strain of virus (Fenner, 1948a), that is, there was a bimodal morbidity curve and a low mortality rate. In the older mice, however, the mortality rate was as high as had previously been found with "Moscow" ectromelia virus spreading through eight-weeks-old mice. The morbidity curve, however, resembled that found with the "Hampstead" strain of virus spreading through eight-weeks-old mice, although the primary peak was somewhat higher. This may be partly accounted for by the fact that there were five sub-clinical cases in the younger group and none in the older. Amongst old mice, therefore, naturally acquired

mousepox due to the "Hampstead" strain of virus spreads with about the same ease as through a herd of eight-weeks-old mice, but is associated with a considerably higher mortality rate.

Suckling mice could not be assembled into standard herds and the spread of mousepox through groups of very young animals had to be studied by a different technique. Experiments were carried out in which four infected female mice were added to groups consisting of two normal mothers and their five-day-old offspring, and two ectromelia-immune mothers and their five-day-old young. Six days later, when the inoculated mice showed early discharging lesions of the feet, one normal and one ectromelia-immune female mouse and their four-day-old young were added. Primary lesions were detected in the young mice from the twelfth day onwards and the first death occurred on the thirteenth day. The results are shown in Table 2.

TABLE 2.

The fate of offspring of normal and ectromelia-immune mice exposed to infection with the "Hampstead" and "Moscow" strains of ectromelia virus.

Strain of virus	Status of mother	Day of entry to herd	Number of young mice	Results				
				Primary lesion seen	Fatal case	Non-fatal clinical infection	Sub-clinical infection	Not infected
"Hampstead"	Immune	0	11	2	1	2	1	7
	Immune	6	5	0	0	0	0	5
	Normal	0	7	1	0	1	4	2
	Normal	6	4	1	2	1	0	1
"Moscow"	Immune	0	14	8	1	7	3	3
	Immune	6	7	2	1	1	4	1
	Normal	0	9	7	1	6	1	1
	Normal	6	6	1	6	0	0	0

The low infectivity of the "Hampstead" strain of virus and the sheltered life led by the suckling young resulted in only 44 p.c. of the young mice contracting mousepox. The results obtained, however, are consistent with those obtained in the experiment with the "Moscow" strain, in which 86 p.c. were infected. In the latter experiment all the normal young mice added on the sixth day died of acute mousepox, while there was only one fatal case among the nine offspring of a normal mouse added on the first day and two among the twenty-one offspring of immune mothers. Within a day of aggregation of the mice a common nest had been built of cotton wool and the young were suckling any available mother. It was found previously that the major part of the antibody passively transferred to young mice was passed in the milk (Fenner, 1948a). The indiscriminate feeding allowed the normal young added on the first day to attain the same immune status as the offspring of the immune mothers, before exposure to mousepox infection. Normal offspring added on the sixth day had no opportunity of acquiring antibody before they were infected and they all died of acute mousepox within ten days of entering the herd. Primary lesions occurred in eighteen of the thirty-one infected mice, and were found on the feet in eight cases and the head in ten. Eight mice suffered subclinical attacks and five did not contract the disease. In only one young mouse was a secondary rash detected.

The Antibody Response in Mice of Different Ages.

In looking for an explanation of the high mortality rate accompanying infection with the "Hampstead" strain of virus in very young and old mice, my first thought was that it lay in differences in the efficiency of the antibody-forming mechanism in mice of different ages. Freund (1930) showed that rabbits less than twenty days old formed antibodies much less readily than did adult rabbits, and Morgan (1941) found a similar deficiency in the production of neutralizing antibody to eastern equine encephalomyelitis in mice less than ten days old at the onset of immunization. No observations are available on antibody production in aged animals, but Sabin, Ginder, Matumoto and Schlezinger (1947) showed that in the absence of previous infection, Japanese more than sixty years old failed to produce either neutralizing or complement-fixing antibodies after immunization with a potent Japanese B encephalitis vaccine.

No survivors were available from inoculated young mice and only one of the inoculated old mice, so data have been drawn from the survivors of the closed epidemic. The suckling mice concerned had been infected when about fourteen days old, and had been protected from death by passively transferred antibody. Histograms have been constructed from the E-AHA titres of the nineteen forty-four-weeks-old and forty-eight eight-weeks-old survivors of the closed epidemic, and the thirty-one infected survivors from the two experiments with suckling mice. As may be seen from Fig. 2, there is no significant difference between the antibody response in the three groups of mice. This result does not conclusively show that defective antibody production is not responsible for the high mortality rates at extremes of age. Only the survivors were tested, and these may be animals in which the antibody response was better than in their companions which died.

Tissue Reactivity in Mice of Different Ages.

It was shown earlier (Fenner, 1948b) that infection with mousepox induced sensitivity to the virus, and this first became evident on the tenth day after infection with a small dose of virus and reached its maximum titre by the fourteenth day. Previous infection with vaccinia virus also conferred sensitivity to ectromelia virus but the reaction was usually less severe. Ten-day-old, eight-weeks-old, and forty-weeks-old mice were therefore inoculated in the left hind foot with either a dilute suspension (about 100 ID_{50}) of "Hampstead" ectromelia virus or a concentrated suspension of vaccinia virus, both preparations being made from infected chorioallantoic membranes. On the fourth, ninth, and fourteenth days mice in each group were inoculated in the right hind pad with a concentrated preparation of ectromelia virus in the form of a saline suspension of mouse liver.

Among the eight-weeks-old mice the responses at the different stages were the same as recorded previously, that is there was no hyper-sensitivity when the test inoculation was made on the fourth day, but hyper-sensitivity was evident by the ninth day and well developed by the fourteenth day. Only one of the suckling mice inoculated with ectromelia virus survived long enough to be tested, but those inoculated with vaccinia virus all survived. None showed an allergic response. The foot inoculated with "Moscow" ectromelia virus became swollen on the same day as in the control animals of the same age, but effective active immunity caused its rapid return to normal and the mice did not become sick. In the old mice, on the other hand, the allergic response appeared if anything slightly earlier than in the eight-weeks-old animals. In the ectromelia-immune mice it subsided by the third day, but in vaccinated animals a multiplication response commenced at about this time. The responses in the groups inoculated fourteen days after the primary inoculation were identical in the old and the eight-weeks-old mice.

The Rate of Multiplication of Virus in Mice of Different Ages.

Investigation of the difference in the virulence of the "Hampstead" and "Moscow" strains of ectromelia virus in eight-weeks-old mice suggested that the higher virulence of the latter might be related to its more rapid multiplication in mouse tissues (Fenner, 1949). It was not possible to carry out direct comparisons of the multiplication rates of the "Hampstead" strain of virus in mice of different ages, but certain inferences can be made from the interval between inoculation of virus in the foot and its isolation from the spleen and the survival times of acutely fatal cases.

As was found in earlier experiments (Fenner, 1949) virus did not reach the spleen of the eight-weeks-old mice until the fifth day. In the suckling mice, however, it was isolated from the spleen on the second or third day and in the old animals on the fourth and fifth day. The virus titres of the livers and spleens of fatal cases were of the same order, viz. 10^9–10^{10} ID_{50} per ml., whatever the age of the mice. These concentrations of virus were reached, with consequent massive necrosis of the liver and spleen, within seven days of inoculation in the suckling mice, but in the old mice, the virus did not reach its lethal titre until the tenth or eleventh day, that is at about the same time as in the occasional fatal case in eight-weeks-old mice.

DISCUSSION.

It is likely that the reasons for the increased virulence of the "Hampstead" strain of virus in suckling mice and old mice are different. An important feature in the suckling animals appears to be the short delay between peripheral inoculation and invasion of the liver and spleen. As in older mice, this interval was shorter with the "Moscow" than with the "Hampstead" strain, for virus was sometimes isolated from the spleen within twenty-four hours of inoculation with the former. With both, however, the delay was much less than in adult mice. The multiplication rate of the virus in the liver and spleen after pad inoculation of a small dose of the "Moscow" strain was, in the single experiment performed, much the same as was found in the organs of adult animals. The shorter time available for antibody production, and perhaps the less efficient antibody production in these young animals, were probably responsible for the almost invariable fatal outcome.

Oerskov and Andersen (1938) obtained similar results in very young rabbits inoculated intradermally with vaccinia virus. In three-day-old rabbits virus was found in the internal organs at the end of twenty-four hours, while in older rabbits there was a delay of two or three days in the lymph nodes before invasion of the liver and spleen. With these very young animals widespread focal necrosis was found, especially in the liver, on about the fifth day. Generalization was slower in eight-day-old rabbits, and in older animals still longer delays occurred, and the titre of virus in the internal organs never reached the same high levels as in the very young. They obtained evidence of more effective antibody production in the older animals, for no virus could be found in the blood or organs of the older rabbits after the eighth day, but the titres in the

organs and the blood of the very young rabbits which survived were still quite high.

With mice the transition between the high mortality of the very young and the low mortality in adult animals infected with "Hampstead" mousepox probably occurred about the third week of life. Some fourteen-day-old mice survived after severe attacks of mousepox, but younger animals (two days old to eight days old) always died. At the end of three weeks the mice had almost gained their adult resistance, and four-weeks-old mice behaved in the same manner as adults when inoculated in the foot with small doses of the "Hampstead" strain of virus.

The reason for the greater susceptibility of year-old animals is not apparent. Such animals are by no means senile, for normal mice will live two years or more (Greenwood, 1928). Invasion of the internal organs occurs at much the same time as in eight-weeks-old mice, and the virus multiplies at much the same rate. Whereas in eight-weeks-old mice infected with the "Hampstead" strain of virus it is only very rarely that virus multiplication proceeds until lethal titres are reached in the liver and spleen, this appears to be the rule in the older animals. The most likely explanation seems to be deficient antibody production, but the available evidence upon this point is unsatisfactory, for sera have been obtained only from survivors and not from the animals which died.

The experiments with mousepox spreading through the young offspring of normal and immune mothers provides an explanation for the fact that a virus which is highly infective and causes uniformly fatal infections in suckling mice can attain endemic status in a mouse colony. Infection contracted by the young under cover of passively transferred antibody was rarely fatal and conferred a high degree of immunity. Animals which were not infected in infancy attained, with the physiological changes that accompanied ageing, a much lower susceptibility to the lethal effects of ectromelia virus.

SUMMARY.

Both the "Moscow" and "Hampstead" strains of ectromelia virus cause a much higher mortality in suckling mice and mice about a year old than in eight-weeks-old mice, whether spreading naturally or inoculated in the foot pad.

Passively transferred antibody from the mother protects suckling mice from fatal infection and good antibody production usually follows infection in such animals.

The delay between pad inoculation of the virus and invasion of the internal organs is much shorter in suckling mice than in adult animals, and this is probably the important reason for the higher mortality which they experience.

Invasion of the internal organs of the older mice occurs at about the same time as in eight-weeks-old animals, but multiplication in the liver and spleen

usually proceeds until lethal concentrations are reached, whereas in eight-weeks-old mice such levels are rarely attained (with the "Hampstead" strain of virus). The explanation is not apparent, but may be associated with deficient antibody production in the older animals.

Acknowledgment. I am indebted to Professor F. M. Burnet for his advice and criticism.

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THE SODIUM SULPHITE "ALBUMIN" FRACTION OF HUMAN PLASMA, AND NOTES ON ITS ESTIMATION

by ALFRED W. STEINBECK

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(Accepted for publication, 26th August, 1948.)

Values accepted as normal for the salt "albumin" fraction of human plasma are greater than those for electrophoretic albumin (Svensson, 1944). The filtrate from the precipitation of plasma protein by 20 p.c. (gm. solute per 100 ml. solvent) sodium sulphite contains electrophoretic globulin as well as albumin (Moore *et al.*, 1944 and 1945). Thus, for globulin precipitation it would appear that the customary 20–22·5 p.c. sulphite concentrations are too low.

While defining solubility curves of plasma proteins against sodium sulphite, the sulphite concentration was extended beyond the previous reference range of Roche *et al.* (1939). The solubility curves, for which the linear plot adopted by Roche *et al.* (1939) was retained, showed in general a displacement in the region of the 25–30 p.c. precipitant. This displacement suggested loss from solution of an insoluble fraction of plasma protein—by inference a globulin fraction. It was decided to repeat these solubility curves to determine whether this displacement was constant and significant.

Evidence is presented to show that 27 p.c. sodium sulphite is a precipitant for total globulin.

METHODS.

Collection of Blood and Plasma.

Venous blood—from a series of apparently normal undergraduates, technicians and hospital patients—was collected into sealed oxalated tubes, using 1·78 mg. lithium oxalate plus 0·8 mg. pot. oxalate per 2 ml. blood. Samples showing any haemolysis were rejected. Equal samples of plasma were pooled.

Globulin Precipitation.

"Globulin" and fibrinogen were precipitated from 0·5 ml. plasma with 15·0 ml. aqueous sodium sulphite, for 1 hour at room temperature. The sulphite concentrations were 10, 15, 20, 22·2, 25, 26·8, 30 p.c. respectively. Nine precipitations were performed in duplicate for each precipitant concentration. Caprylic alcohol was added before filtration. The method was a modification of that of Campbell and Hanna (1937a, b).

Plasma Non-Protein Nitrogen.

Protein-free filtrates were prepared by a modification (Haden, 1923) of the method of Folin and Wu (1919), using half the standard quantity of reagents.

Digestion.

Digestion, as a modified micro-Kjeldahl procedure, was carried out in 12 × 1 inch boiling tubes covered with fitted watch glasses. The digestant was sulphuric acid, with a copper catalyst. Digestion was continued for at least 1 minute after initial lightening of the digest. This lightened digest was cleared by the addition of hydrogen peroxide. Then digestion was further continued for at least 3 minutes.

Digestion of the filtrates was performed in triplicate.

Filtrate from a 22·2 p.c. sulphite precipitation was digested as above, i.e. short-period digestion, and also for 4 hours. Nitrogen estimation of the digest was an estimate of nitrogen recovery.

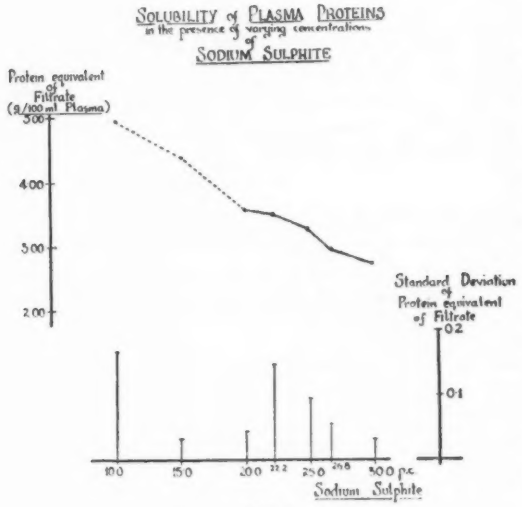

Fig. 1

Nitrogen Estimation.

The Koch-McMeekin (1924) method of direct nesslerization of the digest was followed. Nesslerized standards and digests were compared in a Klett-Dubosq colorimeter, transmitting white light.

In addition, certain aspects of the digestion and direct nesslerization methods were analysed with the object of reducing the systematic error.

After non-protein nitrogen corrections, the nitrogen values were multiplied by 6·25.

RESULTS.

The graph (Fig. 1) of filtrate nitrogen — expressed as protein equivalent—and sodium sulphite concentrations shows the same trend noted in preliminary experiments. The effective slopes of decreasing filtrate nitrogen for the successive 15–20, 20–25, 25– p.c. precipitant ranges are significantly different at the 5 p.c. level of the "t" distribution. The displacement at the 25–26·8 p.c. range is likewise significant. Also, the filtrate nitrogen for the 20–22 p.c. range is approximately 1·16–1·18 times that for the 27 p.c. precipitant filtrate.

The nitrogen recovered with a short-period digestion is significantly greater than with a 4-hour period.

The variance difference for the two digestions is not significant by the "chi-square" distribution.

It was found that the digestion times before and after the addition of hydrogen peroxide were important. Incomplete digestion in a significant number of cases resulted in a fine turbidity of the nesslerized digest. This was read as an apparent nitrogen increase.

For consistent results, direct nesslerization required a rigid technique. The addition of the reagent to the diluted digest by rapid blowing from a specially calibrated pipette with immediate inversion of the incomplete mixture gave consistent results. In a significant number of cases, a fine turbidity of the nesslerized digest resulted from either slow addition

TABLE 1.

Summary of statistics.†

Item	Degrees of Freedom (n–1)	Means (p.c.)	Sum of Squares	Mean Square
Short-period digestion	17	4·299	0·2194	0·0129
4-hour digestion	35	4·236	0·2625	0·0075

$S = 52$ $\bar{x} = 0{\cdot}063$ $Sx^2 = 0{\cdot}4819$

Pooled variance $s^2 = 0{\cdot}009267$ $s_{\bar{x}} = (s^2 (n_1 + n_2)/n_1 n_2)^{\frac{1}{2}}$

$= 0{\cdot}02779$ $t = \bar{x}/s_{\bar{x}} = 2{\cdot}267$*

* = significant at 5 p.c. level.

† q.v. Snedecor, G. W. (1946): Statistical Methods applied to Experiments in Agriculture and Biology. 4th ed. Ames, Iowa: Iowa State College Press.

of the reagent or addition down the tube wall, or from delayed or agitated inversion of the mixture. This turbidity was read as an apparent nitrogen increase.

For a number of series of protein estimations at the "albumin" level the maximum standard deviation (s) was at least 0·15 p.c.

DISCUSSION.

Sodium sulphite and sulphate appear approximately equivalent precipitants for globulin (Campbell and Hanna, 1937b; Isbister, 1946); 26·8 p.c. sulphate appears a complete globulin precipitant for human plasma (Milne, 1947; following Majoor, 1942 and 1947). Preliminary experiments indicated that the displacement of the solubility curve occurs in the range 25–30 p.c. sulphite. Therefore 26·8 p.c. sulphite was chosen as an intermediate precipitant in this range. The evident displacement of the solubility curve in the 25–26·8 p.c. sulphite range indicates exclusion of an insoluble protein fraction. This, by inference from plasma protein solubilities (Cohn, 1942) and electrophoretic analyses (Moore *et al.*, 1944), is part of the α-globulin. This precipitation appears complete with 27–27·5 p.c. sulphite.

From electrophoretic analyses (Cohn *et al.*, 1944; Dole, 1944; Staub and Labat, 1947) the ratio of α-globulin plus albumin to albumin is 1·16–1·18 approximately. The filtrate from 20–22 p.c. sulphite precipitation contains α-globulin plus albumin (Moore *et al.*, 1944). The experimental ratio of the filtrate protein equivalent for 20–22 p.c. range to that for 27–27·5 p.c. precipitant accords with the electrophoretically derived ratio.

Thus for albumin estimation in human plasma it is suggested either that duplicate precipitations be performed with 27 p.c. sulphite at a dilution of 0·5 ml. plasma–15·0 ml. sulphite or (to allow for possible solubility curve displacements) that precipitations be performed with 26·5 and 27·5 p.c. sulphite

and the final results averaged. This method is shorter and less tedious than the 26·8 p.c. sulphate method of Majoor (1942 and 1947) adopted by Milne (1947). Parallel salt fractionations and electrophoretic analyses would constitute critical evaluation of this sulphite precipitant concentration as a complete globulin precipitant.

The solubility graph in the 10–20 p.c. sulphite range shows the same general trend noted by Roche *et al.* (1939), if intermediate precipitant concentrations are not considered. If the molarity of the precipitant is taken as the basis of comparison in salting out processes, then the graph "filtrate nitrogen against molar sulphite" (derived from the accompanying figure) is comparable with those of Wuhrman and Wunderly (1945a, b). These graphs show complete globulin precipitation at 2·05 M H_3PO_4. Similar graphs for abnormal human plasma (Wuhrman and Wunderly, 1945c) indicate that while the albumin displacement of the salting out curves for normal plasma is relatively constant, that for abnormal plasmas is variable (above or below 2·05 M) and not always definite.

Nitrogen recovery with a micro-Kjeldahl digestion has been regarded as incomplete with a digestion period of less than 4 hours (Cook *et al.*, 1945; Reith and Wansink, 1947). Hoch and Marrack (1945) claim a 95·5 p.c. recovery with a digestion procedure comparable to the short period digestion used (see Methods). Statistically a 4-hour digestion results in a lower nitrogen recovery than with the short period digestion. This probably reflects loss of nitrogen, which copper does not appear to enhance with albuminous substances (Reith and Wansink, 1947). Therefore, short period digestion allows a better approximation of the plasma protein nitrogen equivalent.

Dyson and Plant (1943) objected to the large systematic coefficient of variation for the direct nesslerization method. However, direct nesslerization performed with a rigid technique does not introduce appreciable errors in either direction. This has also been noted by Hunter (1947).

Accepting 0·15 p.c. as the maximum standard deviation (S) for the method, then for individual results a significant difference is 0·43 p.c. ($s \times 2\sqrt{2}$). A difference of certainty would be 0·50 p.c. For series, significant differences will be less. Also, it appears that the coefficient of variation (100 s/mean) for the method is 5 p.c. This error is within the biological variation of human plasma proteins, i.e. at the "albumin" level.

SUMMARY.

Evidence is presented which suggests that 27 p.c. sodium sulphite precipitates total globulin in normal human plasma.

For albumin estimation it is suggested that filtrates be prepared with 27 p.c. sulphite; or, 26·5 and 27·5 p.c. sulphite and the final results averaged.

An evaluation of the standard digestion procedure is presented.

A significant difference value between individual results for the method is at least 0·43 p.c.

The coefficient of variation of the method is at least 5 p.c.

Acknowledgments. My thanks are due to Professor C. G. Lambie for his surveillance of this work. To my laboratory assistant B. Campbell, my thanks are also due.

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Wuhrman, F. und Wunderly, Ch. (1947): Die Bluteiweisskörper des Menschen. 1st Ed. Basel: Benno Schwabe & Co. (a) Abb. 8, p. 68. (b) Abb. 6, p. 64. (c) Abb. 7, p. 67.

THE ESTIMATION OF SERUM ALBUMIN BY THE PRECIPITATION OF GLOBULIN WITH SODIUM SULPHITE

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(Accepted for publication 11th August, 1948.)

The separation of the albumin and globulin fractions of serum has always been an unsatisfactory procedure, and it is only with the introduction of electrophoretic analysis that this differentiation has been placed on a sound basis. However, the expensive apparatus and the specialized technical skill required have prevented the use of this method for routine purposes, and for most clinical research.

The standard method that has usually been employed is that introduced by Howe (1921b), using 22·2 p.c. sodium sulphate, in a serum dilution of 1 in 31, and allowing the suspension to stand at 37° C. for 3 hours. It has since been shown that the albumin estimated in this way contains a portion of the α globulins (Luetscher, 1940; Taylor and Keys, 1943; Dole, 1944) and that the error is greatest in abnormal sera with low albumin or high globulin levels. Nevertheless, the information obtained by chemical separation has proved useful, and the Howe method has remained the standard for comparison with other methods in laboratory practice.

Various methods of chemical separation have been introduced in an attempt to simplify the procedure and save time when compared with the standard Howe method. Campbell and Hanna (1937) introduced a method in which 21 p.c. solution of sodium sulphite was the precipitating fluid. With a serum dilution of 1 in 20, the procedure was carried out at room temperature for 10 minutes. They found that their method gave similar results to the Howe method used with a serum dilution of 1 in 20. Robinson, Price and Hogden (1938a, b) introduced two modifications to the Howe method to improve accuracy and speed. Centrifugation was used instead of filtration, which they had shown to have intrinsic errors due to adsorption of albumin on the filter paper (Robinson, Price, and Hogden, 1937). The precipitation was carried out at 25° C. (warm room) instead of at 37° C., and little difference in the results was obtained. Kingsley (1940) added ether to the serum-sodium sulphate mixture and, after shaking, centrifugation produced rapid separation of the globulin, which floated on the top with the ether layer. Another method introduced by Pillemer and Hutchinson (1945), gave results which agreed well with electrophoretic analysis, but required special apparatus for cooling. The globulin was precipitated by 42·5 p.c. concentration of methanol at 0° C., pH 6·7 to 6·9, and ionic strength of about 0·03. Chow (1947) has developed a method of determining albumin by means of a specific precipitin reaction. Cohn and Wolfson (1947) use a combination of precipitating reagents, and more recently (1948) they have used 28 p.c. sodium sulphite as the precipitating reagent, and found it to give results in good agreement with electrophoresis.

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[1] This work was carried out in the Department of Medicine, University of Sydney, and in the Medical Department of the Royal Prince Alfred Hospital.

In the present investigation estimations of albumin have been made on normal and abnormal human sera using the methods of Howe and of Campbell and Hanna, but with a serum dilution of 1 in 31 in both cases.

As shown by Howe (1921a) the use of a serum dilution of 1 in 20 instead of 1 in 30 leads to a decrease of about 0·2 gm./100 ml. in the albumin estimate in the case of certain animal sera. The sodium sulphite method has not been confirmed by other workers as giving results similar to the Howe method, except

TABLE 1.

Clinical condition.	Serum albumin gm./100 ml.		
	Sulphate method	Sulphite method	Diff.
Normal subject, male	4·86	5·13	+0·27
Normal subject, male	4·71	4·77	+0·06
Normal subject, female	4·74	4·83	+0·09
Normal subject, male	4·38	4·59	+0·21
Normal subject, female	4·80	4·86	+0·06
Normal subject, male	5·13	5·39	+0·26
Normal subject, female	4·65	4·77	+0·12
Normal subject, male	5·43	5·52	+0·09
Normal subject, male	4·62	4·65	+0·03
Normal subject, male	4·86	4·90	+0·04
Angina pectoris	4·56	4·53	−0·03
Bronchogenic carcinoma	4·20	4·30	+0·10
Duodenal ulcer	4·80	4·86	+0·06
Myocardial infarction	4·15	4·20	+0·05
Idiopathic epilepsy	4·77	4·89	+0·12
Disseminated sclerosis	4·68	4·74	+0·06
Congestive cardiac failure	4·18	4·15	−0·03
Duodenal ulcer	4·55	4·73	+0·18
Congestive cardiac failure	4·49	4·83	+0·34
Lobar pneumonia	4·23	4·30	+0·07
Thyrotoxicosis	4·68	4·83	+0·15
Anaemia? cause	5·03	5·13	+0·10
Duodenal ulcer	4·44	4·71	+0·27
Congestive cardiac failure	4·25	4·46	+0·21
Thyrotoxicosis	5·13	5·17	+0·04
Ulcerative colitis 1	3·00*	3·07*	+0·07
2	3·08*	3·20*	+0·12
3	2·03*	2·03*	0
4	1·98*	2·07*	+0·09
5	1·85*	1·94*	+0·09
6	2·02*	2·02*	0
7	2·90*	2·95*	+0·05
Ulcerative colitis 1	3·35*	3·10*	−0·25
2	2·67*	2·82*	+0·15
Cerebral arteriosclerosis	4·23	4·17	−0·06
General paralysis of the insane	3·51	3·69	+0·18
"Nephrotic" nephritis 1	1·85*	1·80*	−0·05
2	2·08*	2·33*	+0·25
Colitis	3·81	3·94	+0·13
Infective hepatitis	2·90	2·96	+0·06
Broncho-pneumonia	3·16	3·32	+0·16
Lymphosarcoma of neck	3·87	4·05	+0·18
Chronic nephritis	3·15*	3·15*	0
Tuberculosis of hip joint	3·74	3·70	−0·04
Lobar pneumonia	2·81	3·01	+0·20
Haematemesis	3·73	3·88	+0·15
Iron deficiency anaemia	3·68	3·68	0
Chronic myeloid leukaemia	3·97	4·02	+0·05
Amoebic dysentery	4·15	4·20	+0·05
Congestive cardiac failure	3·12	3·27	+0·15
Sub-acute peritonitis	3·06	3·17	+0·11
"Nephrotic" nephritis	2·64*	2·69*	+0·05
Carcinoma of the pancreas	4·54*	4·56*	+0·02
Means	3·83	3·92	+0·091
Standard error			±0·044

Values followed by an * have had the nonprotein nitrogen estimated and deducted. The remainder of the values have not had the N.P.N. determined, but have had 0·15 gm. deducted which represents a N.P.N. of 24 mg./100 ml. of serum. Serial estimations on the same patient have been numbered.

by Roche, Derrein, and Moutte (1939), who used horse serum—also at a dilution of 1 in 20. A range of abnormal sera was not used, nor were any figures quoted.

METHODS.

All albumin estimations were carried out with duplicate estimations on the filtrate from a single precipitation. 15 ml. of a 21 p.c. solution of sodium sulphite were measured into one test tube and 15 ml. of 22·2 p.c. solution of sodium sulphate into another. To each was added 0·5 ml. of the same serum from pipettes calibrated "to contain", the pipettes being washed out several times in each instance with the contents of the test tubes after mixing. The tube containing the sodium sulphite was kept at room temperature for about 9 minutes, then one drop of capryl alcohol was added and the tube shaken. After standing for a further minute it was filtered through a single thickness of filter paper (Whatman No. 42), the filtrate being refiltered till clear. The tube containing the sodium sulphate was placed in the incubator at 37° C. for 3 hours, and then similarly filtered.

Nitrogen estimation was carried out on 1 ml. aliquot portions of the filtrates by a micro-Kjeldahl technique, employing hydrogen peroxide to assist the digestion, followed by direct Nesslerization (Koch and McMeekin, 1924), and final estimation of the colour in a Klett visual colorimeter. In some cases, the non-protein nitrogen was determined by the same method applied to a protein-free filtrate prepared by the Folin and Wu (1919) technique as modified by Haden (1923). In the remainder of the cases where there was no reason to suppose that the non-protein nitrogen was raised, 0·15 gm. of protein (24 mg. of nitrogen) was subtracted from each result.

RESULTS.

Fifty-three samples of serum were analysed, some being serial determinations on the same subject. The results are set out in Table 1. The figures have been analysed statistically, and the mean by the sulphite method is 0·091 higher than that by the sulphate method, with a standard error of the mean difference of $\pm 0{\cdot}044$. This gives a value for "t" of 2·068 which is just significant at the 0·05 probability level. The mean difference of 0·091 gm. corresponds with an albumin value by the sulphite method of 3·92 gm./100 ml.

DISCUSSION.

As the mean difference between the two methods is of borderline significance statistically, it is obvious that biologically it is of no significance at all. The figures quoted by Campbell and Hanna (1937) have also been analysed. They quoted a series of 54 cases of normal and abnormal sera, but the first 18 of their cases have not been included in this analysis because the addition of capryl alcohol was only made in the remaining 36 cases. The mean by their sodium sulphite method was 0·039 gm./100 ml. higher than their corresponding mean by the Howe method, with a standard error of the mean difference of $\pm 0{\cdot}033$, so that the difference is not significant. Although the series of Campbell and Hanna and the present series were not carried out on the same samples, the differences in the two series were compared, and gave a "t" value of 2·74 which is significant at the 0·05 probability level. There may therefore be a difference between the operators in each series, or the difference between the two series may be accounted for by the different dilutions of the serum.

Despite these differences between the two methods and the two series revealed statistically, it is not considered that it has any significance when applied to clinical investigation, and it can be assumed that the results given by the sodium sulphite method of precipitating serum globulin are similar to those given by the standard Howe method with sodium sulphate.

SUMMARY.

A series of 53 samples of normal and abnormal human serum has been analysed in respect of albumin content by two methods. The globulin has been precipitated by the standard Howe method with 22·2 p.c. sodium sulphate, and by the method of Campbell and Hanna using 21 p.c. sodium sulphite. A serum dilution of 1 in 31 was used in both cases.

The results given by the two methods have been analysed and compared with the results given by Campbell and Hanna. The difference between the two methods in the present series is of borderline significance statistically, as is the difference between the results of the present series and those of Campbell and Hanna.

It is considered that for clinical purposes the two methods give similar results, and that the method of Campbell and Hanna has the great advantages of speed and simplicity.

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MODIFICATION OF VIRUS RECEPTORS BY METAPERIODATE

I. THE PROPERTIES OF IO_4-TREATED RED-CELLS

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Hirst and Hotchkiss (1945) described the action of potassium metaperiodate on erythrocytes resulting in the "destruction of cellular receptors"—the cells were rendered inagglutinable by the PR8 strain of influenza A virus. This observation served as a basis for speculations concerned with the nature and chemical character of receptors. Working on the serum inhibitor for heated viruses Anderson (1948) and McCrea (1948) found that it was destroyed not only by active viruses of the mumps-influenza group, by the "receptor-destroying enzyme" of *V. cholerae* and certain clostridia, but also by KIO_4, a most important finding which actually led to the realization that mucopolysaccharides act as receptors, and to the chemical isolation and purification of the inhibitor from sera and various biological materials (Burnet, McCrea and Anderson, 1947; Burnet, 1947; Burnet, 1948a; Anderson, 1948; McCrea, 1948; Burnet, 1948b, 1948c, 1948d; McCrea, 1949).[2]

As it has been shown that previous enzymatic destruction of receptors on sensitive cells precludes virus-host cell combination, and by this means infection (Stone, 1947a, 1948a, 1948b; Fazekas de St.Groth, 1948a, 1948b), experiments were undertaken to use metaperiodate as a receptor-destroying agent, i.e. as a chemoprophylactic against influenza. While looking for the minimal effective dose in mice by the excised lung technique, it soon became apparent that there were basic differences between the action of "receptor-destroying enzymes" and KIO_4. The enzymes contained in bacterial culture filtrates inactivate receptors completely: cells treated with these preparations do not bind virus at all, and

[1]This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

[2] Some of the experiments reported in the first three papers of this series have since been repeated and the results confirmed by G. K. Hirst (1948); J. exp. Med. 87, pp. 301 and 315. Due to the use of excessive amounts of IO_4 and the red-cell agglutination test as the only criterion of the presence *and state* of receptors, the action of this chemical has been again misinterpreted, Hirst's contention being that the viral enzyme and periodate not only have a common substrate, but also a similar mode of action. This apparent parallelism can only hold when investigations are restricted to that narrow range of periodate action which is termed "the zone of destruction" in this paper. It appears that Hirst's studies have been confined to reactions within this zone.

adsorbed influenza virus is quantitatively removed by their action from the cellular surface.

With KIO_4 on the other hand comparable results are obtained only with very large quantities, smaller amounts of the chemical only *modify* the behaviour of suitable cells: virus is adsorbed to them, but spontaneous elution does not occur, nor does artificial removal by receptor-destroying enzymes.

The phenomenon was studied in some detail; the present paper reports experiments performed on red-cells.

MATERIALS AND METHODS.

Normal saline. 8·50 gm. NaCl in 1,000 ml. distilled water.

Calcium saline. 1·00 gm. $CaCl_2$, 8·50 gm. NaCl, 1·203 gm. H_3BO_3 and 0·052 gm. $Na_2B_4O_7.10\,H_2O$ in 1,000 ml. distilled water (pH = 7·2).

Citrate saline. 20·00 gm. sodium citrate and 8·50 gm. NaCl in 1,000 ml. distilled water.

Glycerol saline. 9·20 gm. glycerol and 8·50 gm. NaCl in 1,000 ml. distilled water, i.e. 0·1 M glycerol in normal saline. Further dilutions of this stock solution were invariably made in normal saline. Concentrations will be expressed in the molarity of glycerol, e.g. 1:10 dilution of the original glycerol (0·1 M) saline will be designated as glycerol (0·01 M) saline.

Metaperiodate. 2·300 gm. of crystalline, chemically pure KIO_4 was dissolved in 1,000 ml. of normal saline. Further dilutions were always made in normal saline, the concentrations will be expressed in the molarity of KIO_4. The stock solution is of 0·01 M strength.

Red-cells. The venous blood obtained from healthy adult animals was mixed with citrate saline, centrifuged and the cells washed in two changes of normal saline. The packed erythrocytes were stored at 4° C., and no cells older than 36 hours were used.

For the titrations 1 or 2 p.c. suspensions were prepared in normal saline. As is the routine of this Institute, all fowl-cells were classified according to their sensitivity to various agglutinins and inhibitors, and only the most suitable for a particular test was used in special titrations. Human, rabbit and sheep red-cells were used besides fowl-cells.

Virus. All strains were grown in embryonated hen's eggs according to the standard methods described in Beveridge and Burnet (1946), and had numerous egg passages since their isolation.

"WS" and "Melbourne"—Classical strains of influenza A.

"IAN" and "BEL"—A-strains isolated in the "O" phase from epidemic influenza patients, and used in the experiments as infected amniotic fluids containing virus in the "O", intermediate and "D" phase respectively.

"Swine"—Shope's strain "15" of swine influenza.

"LEE" and "Mil"—The classical and a recently isolated strain of influenza B.

The allantoic or amniotic fluids were pooled and stored at 4° C.; they are referred to in this paper as "active." "Heated virus" was prepared by heating the fluids for 30 minutes at 56° C. in the water-bath.

Receptor-destroying enzyme (RDE). The 4Z strain of *V. cholerae* was grown on soft agar plates for 16 hours at 37° C. The fluid was pressed out of the agar held in a gauze bag, passed through a Büchner funnel, and the filtrate heated at 55° C. for 40 minutes in the presence of excess calcium at pH 6·0. The precipitate was removed by centrifugation and the supernatant stored at 4° C. in the refrigerator. These preparations are referred to

in this paper as RDE; they had a yellowish colour, were completely transparent, and of a pH between 5·9 and 6·5. Their titre—as estimated by the standard technique described below—was about 1,600.

For further details of the properties and preparation of RDE see Stone (1947b), Burnet and Stone (1947), and McCrea (1947).

Purified inhibitor. This was prepared by the method of McCrea (1949) from ovarian cyst fluids. The preparation, a greyish-white powder, inhibited red-cell agglutination by 100,000,000 agglutinating doses of heated LEE virus per gm. of dry weight. In the experiments the purified inhibitor was made up to suitable dilutions in normal saline. The activity will be expressed by the inhibitory titre obtained in the standard titrations (*v. infra*).

For full description of method see the original paper by McCrea (1949).

Anti-virus sera. Sera of vaccinated rabbits, ferrets and fowls were used, as well as convalescent human sera after natural disease. As controls sera of normal animals and the first bleedings of the human patients were included in the experiments.

Anti-RDE serum. This was from rabbits immunized with an emulsion of eluted enzyme—liquid paraffin—lanolin base. The properties of this serum are described in Burnet (1949).

Titration of virus haemagglutinin. The routine technique used in this laboratory for estimating haemagglutinin titres had to be slightly modified, as in many cases the samples contained RDE in addition to virus. As the activity of this enzyme on red-cells is greatly reduced in the absence of calcium ions, all dilutions were made in citrate saline. As seen in previous work, virus suspensions gave the same titre in citrate saline as in normal saline, and 10 A.D. of virus could be accurately assayed in media containing 1,000 inhibitory doses of RDE.

Doubling dilutions were made in 0·25 ml. citrate saline and 0·25 ml. of a 1 p.c. red-cell suspension added. Readings were made an hour later by observing the pattern of the settled cells. 1 A.D. is the amount of virus which causes "+" (partial) agglutination of 0·25 ml. 1 p.c. fowl-cells.

Titration of RDE. The method of Burnet and Stone (1947) was used. Doubling dilutions of the samples were made in 0·25 ml. of calcium saline and an equal amount of 1 p.c. fowl-cells added. The racks were thoroughly shaken and placed in a water-bath at 37° C. for 30 minutes. At the end of this period a drop containing 10 agglutinating doses of active Melbourne virus was added, the racks shaken and put back for another 30 minutes in the bath. The end-point was taken as the "+" pattern of settled cells.

Inhibitor titration. As the samples frequently contained RDE, all titrations were made in citrate saline. Doubling dilutions were made in a volume of 0·25 ml. and a drop of diluted allantoic fluid containing 5 agglutinating doses of heated LEE virus was added. After 30 minutes' standing at room temperature 0·25 ml. of a 1 p.c. fowl-cell suspension was added and readings made an hour later. In these titrations only "inhibitor-sensitive" cells were used, and the titres are expressed as the reciprocal of the dilution giving "+" agglutination.

Antibody titration. The technique of Burnet *et al.* (1942) was used with slight modifications. The samples were diluted in twofold steps in 0·25 ml. citrate saline and a drop containing 3 agglutinating doses of active virus was added. The racks were left for 30 minutes at room temperature, then 0·25 ml. of 1 p.c. fowl-cells added and readings made after another period of 60 minutes. The end-point was taken as "+" agglutination, and titres are expressed as the reciprocal of the dilutions giving this pattern against 3 A.D. In all antibody titrations fowl-cells of "low inhibitor-sensitivity" were used.

If not stated specially, all experiments and titrations were made at room temperature ranging from 19° C. to 24° C. The reagents were stored at 4° C. in the refrigerator and checked from time to time. Only those of full activity were used.

EXPERIMENTS.

In a series of preliminary experiments the properties of periodate treated red-cells were tested by routine laboratory methods.

It was found that the osmotic resistance of erythrocytes has been slightly reduced by KIO_4 treatment with doses higher than 2 mg./gm. RBC. Similarly, this was the limiting amount which caused a small, hardly significant reduction in the sedimentation rate of these cells. As an incidental finding it was noticed that Ca-ions agglutinated periodate-treated cells in relatively low concentrations.

Whether these effects are due to KIO_4, its degradation products or compounds produced by its action was not further investigated as it did not seem to be relevant to the present work. As experiments making use of differences in the sedimentation of red-cells treated with various receptor-destroying and modifying agents will be reported in a separate paper, the full description and discussion of these results is deferred till then.

Behaviour of KIO_4-Treated Cells in Relation to Viruses.

In the standard experiments 10 p.c. human red-cell suspensions were treated with KIO_4. The chemical was used in concentrations ranging from M/100 to M/100,000, all dilutions being made in normal saline. The cells were treated with amounts of periodate from 23 mg./gm. of packed RBC to 0·022 mg./gm. Equal volumes (0·50 ml.) of the ingredients were measured into calibrated centrifuge tubes, thoroughly mixed and kept for 15 minutes at room temperature. At the end of this period 1·0 ml. of glycerol (0·01 M) saline was added, the cells spun down, washed in two changes of glycerol (0·01 M) saline, and finally made up to a 10 p.c. suspension in normal saline. The washing was made with pre-chilled fluids in the cold-room at temperatures ranging from 0° C. to 4° C. Subsequently 0·50 ml. of chilled allantoic fluid virus was added to the KIO_4-treated cells. After 30 minutes in the cold the tubes were centrifuged and 0·05 ml. of the supernatant removed. The sample taken (''a'') was tested for its agglutinin titre. The difference between the amount of agglutinin originally contained in the 0·50 ml. of allantoic fluid and the amount present after 30 minutes' contact with the cells will be referred to as ''adsorbed agglutinin''. Following this the tubes were placed in a water-bath at 37° C. and left there for 240 minutes. At the end of this period the cells were spun down, 0·25 ml. of the supernatant removed (sample ''b''), and replaced by 0·25 ml. of RDE (titre 1,500). The racks were returned to the water-bath for another 30 minutes, centrifuged and sample of the supernatant (''c'') tested for its agglutinin content. The extent of ''spontaneous elution'' is expressed by the difference in agglutinating doses between ''a'' and ''b''; and ''c'' minus ''b'' gives the amount of virus removed from the cells by ''artificial liberation''. Correction has to be made of course for the amount of virus removed in sample ''b''. In control experiments it was found that spontaneous elution was practically complete by the end of the third hour with the amount of reagents used in this set-up, so the quantity recorded in the Tables as ''spontaneous elution'' could be regarded as maximum, i.e. equilibrium value.

From Table 1, the protocol of a typical experiment with Melbourne A virus, it can be seen that KIO_4-treated red-cells adsorb virus to varying degrees, according to the quantity of periodate with which they were treated. There is

TABLE 1.

Adsorption-elution experiment with IO_4-treated red-cells.
(Melbourne A virus.)

	Experimental readings (Agglutinin titre of removed sample)												
	Pre-treatment (mg. KIO_4/gm. RBC)											Controls	
	23·00	11·50	5·75	2·88	1·44	0·72	0·36	0·18	0·09	0·04	0·02	No IO_4^-	No cells
"a"	160	160	120	40	—	7	—	—	—	—	—	—	160
"b"	160	140	100	30	—	—	—	—	10	27	80	60	200
"c"	140	120	70	30	—	—	—	7	17	60	140	160	140
	Calculations (Percentage of bound agglutinin)												
"Bound agglutinin"	0	0	25	75	100	98	100	100	100	100	100	100	0
"Spontaneous elution"	0	1	37	81	100	100	100	100	94	85	50	63	0
"Artificial liberation"	0	3	47	81	100	100	100	97	90	63	6	0	0
Zones	destruction		modification and partial destruction		modification			partial modification			nil		

— = <5.

no measurable adsorption from 10 mg. KIO_4/gm. of packed RBC upwards; this will be called the *zone of destruction*. From 10 mg./gm. to 2 mg./gm. less virus is bound than by the untreated control cells, and in this range neither spontaneous elution nor artificial liberation occurs (*zone of modification with partial destruction*). Between 2 mg./gm. and 0·2 mg./gm. all of the virus is adsorbed and none is removable by the action of RDE: *zone of modification*. Amounts smaller than 0·2 mg./gm. induce *partial modification*, and under 0·05 mg. KIO_4/gm. RBC there is no appreciable effect.

During the periodate treatment haemolysis was observed in the tubes containing concentrations of the chemical higher than M/400. As haemoglobin does not interfere with agglutinin titrations and stromata of red-cells adsorb virus as well as intact cells, the adsorption-elution experiment was continued even with the haemolysed samples. During the washings with glycerol saline, slight (< 30 p.c.) haemolysis was noted even in the tubes containing M/800 KIO_4 previously. The nonhaemolysed cells behaved to all appearance like the untreated control cells, and due to the large excess of virus used in the experiment heavy clumps of agglutinated cells were formed which sank rapidly to the bottom of the tube. During the period of spontaneous elution the racks were repeatedly shaken. After the first hour cells treated with amounts of KIO_4 less than 2 mg./gm. did not clump together immediately after shaking, while those treated with larger amounts formed macroscopically well-distinguishable aggregates even after strong mechanical agitation, and settled soon leaving an almost clear supernatant. It is to be understood that all cells gave a pattern of agglutination when settled, but there were marked differences in the rate of sedimentation which roughly paralleled the amount of bound agglutinin.

After the action of RDE all cells treated with "modifying" and "partially modifying" doses of KIO_4 became stable as did the untreated control cells. Those falling in the zones of destruction or modification with partial destruction were not stabilized and gave definite agglutination patterns even after RDE-treatment.

These experiments indicated that in all probability the action of periodate in the zone of modification is so to alter the receptor substance that it is no longer susceptible to the enzymatic action of RDE or virus. The possibility that entirely new "receptors", from which liberation of virus is impossible, are formed by periodate action can be excluded by the following experiment.

To destroy normal receptors, cells were treated with RDE (0·5 ml. 10 p.c. human RBC + 0·5 ml. RDE, 30 minutes at 37° C.), and washed in three changes of citrate saline. The further treatment of these cells was exactly the same as described in the experiment above (KIO_4-virus-RDE). It was found that irrespective of the amount of KIO_4 used, the cells were not agglutinated and did not adsorb any virus: after enzymatic destruction of "receptors" periodate had no effect on red-cells, it did not produce receptors *de novo*. Further experiments furnishing additional proof of the same point, but whose main bearing is on other aspects of virus-cell combination will be described in another paper of this series.

The behaviour of KIO_4-treated cells has been tested with several virus strains of the influenza group. The results obtained were essentially the same with each of them: the different zones of receptor alteration were clearly shown in all experiments, although the amount of periodate necessary to produce the effects varied in a characteristic manner from strain to strain, and no complete

destruction of adsorptive power could be observed when viruses from the far end of the receptor gradient were used. A full account of this work and of its bearing on the significance of the receptor gradient (Burnet, McCrea and Stone, 1946) will be given in a later paper.

It is of interest that sheep-cells which were repeatedly reported as non-agglutinable by influenza viruses are readily agglutinated after KIO_4 treatment and give as high titres as human or fowl erythrocytes. Similarly, work in this Institute (Anderson, 1947c; Stone, 1948c) has indicated that viruses in the intermediate phase between "O" and "D" (Burnet and Bull, 1943; Burnet and Stone, 1945), which agglutinate fowl-cells only to a very low titre, if at all, give titres comparable to those obtained with human-cells if the fowl-cells are pre-treated with KIO_4. A summary of these experiments is given in Table 2, the discussion of their theoretical and practical significance follows at the end of the paper.

TABLE 2.

Differences in agglutinability of IO_4-treated and normal red-cells.

(Titres are expressed as reciprocal of dilution giving "+" agglutination.)

		LEE	MMF	Swine	CAM O	CAM ω	CAM δ
Sheep-cells	KIO_4 treated	160	120	160	—	—	—
	untreated	<10	<10	<10	—	—	—
Fowl-cells	KIO_4 treated	280	240	—	<10	60	120
	untreated	280	240	—	<10	<10	40
Human-cells	untreated	240	240	—	120	80	160

— = not tested.

PROPERTIES OF PERIODATE-VIRUS-RDE TREATED RED-CELLS (PVR-CELLS).

The findings that KIO_4-treated cells adsorbed virus, and that RDE—though incapable of removing virus from the agglutinated cells—stabilized them, were of much interest. The properties of these cells immediately bring to mind the virus-coated cells prepared with the viruses of Newcastle disease of fowls (Burnet and Anderson, 1946; Anderson, 1947a) and mumps (Burnet, 1946b; Lind, 1948). Since cells similarly coated with influenza virus could not be prepared by the previous technique but would be of great practical value, the behaviour of PVR-cells was studied in detail.

After a series of comparative experiments the following simple method was devised to provide cells maximally coated with virus and stable under all normal conditions:

1. x ml. of packed fowl red-cells + x ml. KIO_4 M/800 in normal saline; mix and leave for 10 minutes at room temperature.

2. Add at least $10x$ ml. allantoic fluid with an agglutinin titre higher than 500, mix thoroughly and leave for 10 minutes at room temperature. (If the allantoic fluid had a lower titre, the difference has to be made up by using a proportionately larger amount of fluid.) By the end of this period the heavily agglutinated cells are almost completely settled; to reduce the volume in which the reaction takes place, most of the clear supernatant may be pipetted off without previous centrifugation.

3. Add x ml. RDE (titre approximately 1,500 by the standard technique of titration), shake and place tube for at least 15 minutes in water-bath at 37° C.

4. Centrifuge, remove and discard the supernatant, wash cells in citrate saline (two changes of approximately 10 x ml. Make up 1 p.c. PVR-cell suspension in citrate saline.

The preparation takes approximately 45 minutes; all times given are the minimum required—an over-treatment with any of the reagents does not influence the final results. PVR-cells prepared by this technique have the following properties.

(a) *Agglutinin on Stable Cells.*

PVR-cells are stable (not agglutinated) in suspension at temperatures ranging from 0° C. to 37° C.; higher temperatures were not tested. But they agglutinate all normal red-cells which the virus with which they are coated would agglutinate. This is shown in the following experiment:

0·25 ml. of 1 p.c. PVR-cells were diluted in twofold steps in citrate saline. To each tube 0·25 ml. of a 1 p.c. red-cell suspension was added, the racks shaken and left standing at room temperature for 60 minutes. Readings were made by observing the pattern of settled cells, partial agglutination being taken as end-point. Tests were performed with fowl and human PVR-cells, and human, rabbit, fowl and sheep cells were used as untreated indicator cells. The results with LEE-coated PVR-cells are shown in Table 3.

TABLE 3.

Agglutination of normal cells by PVR-cells.

(PVR-cells coated with LEE virus.)

Indicator cells	Dilution of PVR-cells							Control: saline instead of indicator cells
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1
Human	++	++	++	++	± ±	±	tr	— —
Fowl	++	++	++	++	± ±	+	±	— —
Rabbit	++	++	++	++	± ±	+	±	— —
Sheep	tr	— —	— —	— —	— —	— —	— —	— —

(b) *PVR-Cells are not Agglutinated by Influenza Viruses.*

0·25 ml. 1 p.c. PVR-cells and 0·25 ml. saline containing 100 A.D. of the test virus were mixed and the degree of agglutination read 60 minutes later.

Cells coated with the following viruses (WS, Melbourne, Bel-D, Swine, LEE, Mil) were tested against each virus. In no case was there any agglutination. If PVR-cells were prepared from erythrocytes sensitive to vaccinia-mouse pox-lipid agglutination, they were agglutinated by these agglutinins. All PVR-cells are agglutinated by T-agglutinins, but not by the members of the influenza group.

(c) *PVR-Cells Contain Active Virus Enzyme on their Surface.*

To test whether the receptor-destroying activity of the adsorbed virus particle is retained, the following experiments were performed.

0·25 ml. of purified ovarian cyst mucoid, previously diluted to give a titre of 320 against 5 A.D. of heated LEE virus was added to 0·05 ml. of 5 p.c. PVR-cells. Three controls contained respectively instead of PVR-cells the same volume of (1) normal saline, (2) 5 p.c. cells treated with LEE virus and RDE but without preceding KIO_4-treatment, and (3) active LEE virus. The mixtures were kept for 60 minutes at 37° C. in the water-bath. Following this they were centrifuged, 0·20 ml. of the supernatant was removed and heated to 100° C. for 5 minutes. By this treatment the virus present in the sample is destroyed, but not the inhibitor. After cooling the inhibitor content was determined by the routine technique of titration. Table 4 shows the results.

TABLE 4.

Destruction of soluble inhibitors by PVR-cells.

(PVR-cells coated with LEE virus.)

Previous treatment of inhibitor	Inhibitor titration								Reduction of titre p.c.
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	Titre	
PVR-cells	++	++	++	++	++	++	++	<5	100
Normal saline	−−	−−	−−	−−	−−	±	±±	240	0
VR-cells	−−	−−	−−	−−	−−	±	±±	240	0
LEE virus	++	++	++	++	++	++	++	<5	100

It is evident that PVR-cells have the same capacity to destroy the inhibitory effect of cyst mucoid as the virus with which they have been treated, while cells treated in the same way with virus and RDE but not previously with KIO_4 do not exhibit any destructive action.

A second experiment showed that the virus on PVR-cells was equally active in destroying the receptors on normal erythrocytes. The aim of this experiment was to treat normal human red-cells with PVR-fowl cells, and then to test whether or not the human-cells still have receptors—are agglutinable by virus.

The reagents used were:

F—1 p.c. fowl red-cells in citrate saline;
PVR—1 p.c. PVR-cells prepared from F, in citrate saline;
H—1 p.c. human group O red-cells in citrate saline;
aL—anti-LEE rabbit serum 1:5 in citrate saline, final titre 60;
Mel—allantoic fluid containing "Melbourne" virus, titre 500.

The reagents were brought together for suitable periods of time at the end of which samples of 0·05 ml. were taken and diluted 1:10 in citrate saline. A drop of these dilutions was put in an ordinary RBC counting chamber and an "agglutination count" made in a standard volume of 0·1 mm^3. A count was made of the total number of cells designated as *F* and *H* respectively, and then a second count in the same area determining the number of cells touching at least one other cell. For fowl-cell touching fowl-cell the symbol *FF* is used, human touching human is *HH*, and human in contact with fowl is *HF*. The total

number of cells present in the standard area will be taken as 100 p.c., and the proportion of cells in contact with other cells will be expressed as percentage.

The simple controls—PVR, F, H suspensions—contained less than 5 p.c. cells touching each other. As these suspensions were found to be stable in the ordinary macroscopic tests, "less than 5 p.c." is considered as the equivalent of "no agglutination". After adding Mel virus to the tubes, F and H were heavily agglutinated, this was shown as more than 30 p.c. *FF* or *HH* cells respectively under the microscope. PVR-cells were not agglutinated by the virus (less than 5 p.c. *FF*). On the other hand anti-LEE serum agglutinated PVR-cells, but not normal F or H cells; this was equally clearly seen by macroscopical and microscopical observation.

In the experiment proper PVR and H, and as a control F and H were brought together for 120 minutes at 37° C. At the end of this time human-cells were agglutinated by PVR-cells (more than 20 p.c. *HF*), but not by normal fowl-cells (less than 5 p.c. *HF*). Subsequently, to separate the PVR-cells from the human-cells, anti-LEE serum was added to the tubes. The PVR-cells were agglutinated to each other, exactly as in the preliminary experiments, and the human-cells which they were previously touching, stood separately in the counting chamber. The serum had no effect on the mixture of F and H cells. Finally, 100 agglutinating doses of Mel virus were added to each tube. The subsequent cell count showed that the human-cells treated with PVR-cells were not agglutinated by the virus, while those treated with normal fowl-cells had not lost their receptors and were heavily agglutinated.

The results of this experiment prove that the adsorbed virus retains its receptor-destroying activity against red-cells as well as against soluble inhibitors.

(d) *PVR-Cells React with Specific Antibody.*

(1) *Direct agglutination by antisera.* 1:10 dilutions of human and animal sera were heated to 62° C. for 5 minutes and after cooling absorbed with RDE-treated fowl-cells to remove T-agglutinins. The sera were further diluted in citrate saline and mixed with an equal volume of 1 p.c. PVR-cells. The test was read as an ordinary agglutinin titration. Table 5 shows the results obtained with active LEE- and Mel-coated cells. For the sake of comparison estimations of antihaemagglutinin content by the orthodox technique (see Methods) are included.

TABLE 5.

Direct antibody titration with PVR-cells.

(Description in the text.)

Anti-body against	Method of assay	Sera									
		Anti-LEE			Anti-Mel			Normal			
		H	Fo	Fe	Fo	Fe	R	H	Fo	Fe	R
LEE	PVR	3,000	2,000	3,000	120	250	400	250	100	400	400
	orthodox	280	160	200	15	30	30	20	15	27	30
Mel	PVR	250	200	480	6,400	4,800	4,000	480	250	400	400
	orthodox	30	15	40	800	480	320	40	20	40	60

H = human sera; Fo = fowl sera; Fe = ferret sera; R = rabbit sera.

It is to be seen that PVR-cells are specifically agglutinated by antisera against the virus they are coated with. The titres are of the order of thousands, while nonspecific agglutination gives titres in the hundreds. The method has no advantage over the ordinary technique of antibody titration, as it is as sensitive to nonspecific agglutination as the other to nonspecific inhibition. Actually the ratio of the titres obtained by the two methods is very nearly constant.

(2) *Specific inhibition of ASC-agglutination by antisera.* As PVR-cells agglutinate normal cells (see above: agglutinin on stable cells), it was supposed that homologous antibody would inhibit this agglutination. To test the validity of this assumption, the following basic experiment was performed.

PVR-cells were diluted in citrate saline to contain 2 A.D. in a standard drop (0·04 ml.). This amount was added to 0·25 ml. of the serum dilutions, and the mixture kept for 30 minutes at room temperature. At the end of this period 0·25 ml. of 1 p.c. fowl-cells (inhibitor insensitive) were added and the degree of agglutination read after 60 minutes' standing.

As seen from Table 6, the test gives high titres with specific antibody, but is still slightly sensitive to nonspecific inhibition. To get rid of this disturbing factor the sera were first diluted 1:2 in normal saline and heated to 62° C. for 5 minutes to destroy the nonspecific inhibitor. After cooling an equal amount of RDE was added, the tubes kept for 15 minutes in a water-bath at 37° C. and then diluted further in citrate saline. By this means the mucoid inhibitor was destroyed as well. The further steps were the same as in the first experiment.

TABLE 6.

Antibody titration by ASC-inhibition.

(Description in the text.)

Anti-body against	Pre-treatment	Sera Anti-LEE			Sera Anti-Mel			Normal			
		H	Fo	Fe	Fo	Fe	R	H	Fo	Fe	R
LEE	nil	640	480	480	30	40	60	40	15	40	60
	62° C. 5′ and RDE	480	400	480	—	—	—	—	—	—	—
Mel	nil	80	40	100	960	480	400	60	20	40	100
	62° C. 5′ and RDE	—	—	—	960	480	400	—	—	—	—

(Sera same as in Table 5.) — = <10.

This technique seems to be superior to other methods of antibody titration in so far as it is sensitive only to specific antibodies. However, its performance takes almost twice the time the orthodox method requires, and thus its practical value will be restricted to comparative titrations with small antibody rise, or to assay sera against inhibitor sensitive virus strains. Studies are under way and a critical evaluation of the method will be given in a later communication.

(3) *The action of inhibitory mucoids on PVR-cells.* The purified mucoid of ovarian cyst fluids (McCrea, 1949) was diluted in twofold steps in 0·25 ml. normal saline and mixed with an equal amount of 1 p.c. PVR-cells. The racks were kept for 60 minutes in the refrigerator at 4° C. and the test read as an ordinary agglutinin titration. The concentration of inhibitor is expressed by its titre as assayed by the routine technique described under Methods. After the reading has been made the racks were shaken and placed in a water-bath at 37° C. for another 60 minutes when the pattern of settled cells was read again. The PVR-cells used in this experiment were coated with active Mel, Swine, LEE and heated LEE viruses respectively. Table 7 gives the results.

TABLE 7.

Agglutination of PVR-cells by inhibitory mucoid.

PVR-cells coated with	Test performed at	Concentration of inhibitor											
		10,240	5,120	2,560	1,280	640	320	160	80	40	20	10	5
Active LEE	0° C.	++	++	++	++	++	++	++	++	±±	+	—	—
	37° C.	++	±±	+	±	tr	tr	—	—	—	—	—	—
Heated LEE	0° C.	++	++	++	++	++	++	++	++	++	±±	+	±
	37° C.	++	++	++	++	++	++	++	++	++	++	±	—
Active Mel	0° C.	++	++	++	++	++	++	++	++	++	+	tr	—
	37° C.	++	+	tr	—	tr	—	—	—	—	—	—	—

It will be seen that all PVR-cells were agglutinated by the inhibitory mucoid to high titres in the cold. However, after an hour at 37° C. only the heated LEE-coated cells gave the same end-point, while there is a significant drop in titre in all tests performed with active virus-coated PVR-cells. The phenomenon is easily explained by the fact that active viruses destroy the inhibitor which is adsorbed to them and forms bridges between the virus-coated cells, thus causing agglutination.

As seen in control experiments, PVR-cells were not agglutinated by mucoids which were not inhibitory for heated LEE virus, or whose inhibitory capacity has been previously destroyed by RDE.

The Properties of KIO_4-RDE Treated Cells.

All experiments done with PVR-cells were repeated with KIO_4-RDE treated cells. The preparation of these was the same as that of PVR-cells with the difference that the second step (treatment with virus containing allantoic fluid) was left out.

It does not seem to be necessary to give a detailed description of these experiments as all results were negative. The cells had merely the properties of RDE-treated cells. They were stable in suspension, but did not agglutinate normal cells. Neither did they destroy receptors or soluble inhibitors, nor were they agglutinated by specific anti-RDE serum or mucoids. As all RDE-treated cells, they were agglutinated to high titre by T-agglutinins.

DISCUSSION.

A study has been made of the phenomenon of receptor modification by dilute KIO_4. Erythrocytes which normally adsorb the viruses of the influenza group are altered by periodate treatment so that the adsorbed particle is firmly held by the cell. Neither spontaneous elution occurs, nor is artificial liberation possible by the use of the "receptor-destroying enzyme" (RDE) of *V. cholerae*, an agent known to remove adsorbed active or heated virus quantitatively from red-cells, from the respiratory surface of mouse lungs or from the lining membrane of allantoic cavities.

The virus bound to periodate-treated cells has not lost its agglutinating and receptor-destroying activity. This was shown with equal clarity in experiments where normal cells were agglutinated and their receptors destroyed, and in others where the inhibitory titre of ovarian cyst fluids was reduced to zero by virus-coated cells as readily as by free virus.

Neither is the antigenic structure changed: periodate-virus-RDE treated cells (PVR-cells), stable in suspension, are agglutinated to high titres by specific but not by heterologous antisera. These PVR-cells give an alternative method of antibody titration and an improved means of serological cross-absorption experiments: the removal of type-specific antibody and excess antigen is performed by simple low speed centrifugation, the antigen being firmly bound to and sedimented with the red-cells.

Thus all properties of the virus which can be tested *in vitro* are unaltered, yet there is no elution from KIO_4-treated cells. Any hypothesis which accepts the enzyme as the active agent in the phenomenon of virus-cell combination is of no help in explaining the findings reported in this paper. The virus has been shown to retain all its characteristic properties including the capacity to destroy normal receptors, but on cells treated with modifying doses of metaperiodate no elution can occur. The substrate has thus been rendered insusceptible to enzymatic action without loss of the adsorptive power of the cell receptor for virus. This is in one sense the converse of the finding that viruses whose receptor-destroying enzymes have been inactivated by various physical and chemical means are adsorbed to normal cellular receptors but no spontaneous separation occurs. Thus the mechanism of combination with the cell seems to be completely independent of the mechanism of elution, and by "modifying" doses of metaperiodate only the latter is affected.

If virus-host cell combination was attributed to specific adsorption between

surfaces of colloidal dimensions (virus and cellular receptor area), and elution to the progressive alteration of the receptor area by the "receptor-destroying enzymes" contained in the viral surface or in *V. cholerae* culture filtrates, the modification of receptors by metaperiodate is readily explained. Receptor areas containing KIO_4-modified substrate for RDE still adsorb the virus particle, but enzymatic elution is not possible.

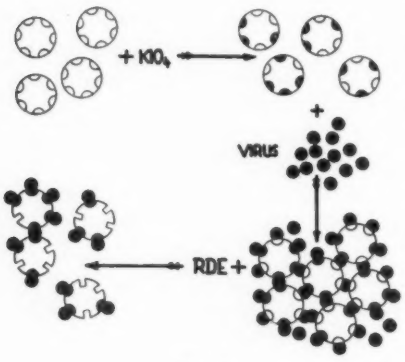

Fig. 1.

In the light of the reported experimental results the alternative view on virus-host cell combination proposed by Anderson (1947b) is accepted. This hypothesis, based on work with heated viruses and partly on findings reported in this paper, is not only in good agreement with all available data concerning the initial phase of virus-cell combination including a set of facts incomprehensible on the basis of the "enzyme-substrate combination hypothesis", but also provides a plausible theoretical foundation for the receptor gradient, a phenomenon until now as puzzling as it has been reproducible.

In Fig. 1 a schematic representation of KIO_4 action on red-cells is given. Periodate modifies receptors by acting upon the substrate for RDE contained in them. Thus the virus adsorbed to modified receptors does not elute spontaneously, neither is it removed by RDE. In spite of being coated with virus, these PVR-cells are stable in suspension: there are no free receptors on their surface, the modified ones being blocked by virus and all the rest having been destroyed either by KIO_4 or RDE.

The reactions of PVR-cells are shown in Fig. 2.

The virus bound to the surface of PVR-cells combines with the receptors of normal cells and causes by this means their agglutination (I.). The specific agglutination of PVR-cells themselves by homologous antibody is the magnified picture of the assumed micro-agglutination occurring when virus and antibody are

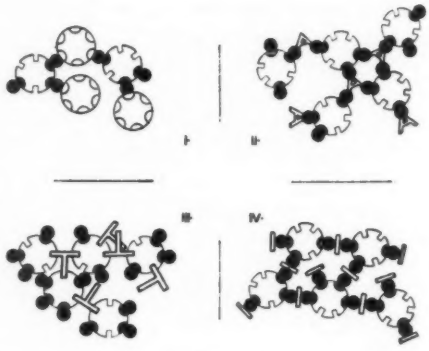

Fig. 2.

brought together (II.). Similar is the mechanism of PVR-cell agglutination by inhibitory mucoids (IV.). The T-agglutination on the other hand (III.) is not specific for PVR-cells: all cells treated with RDE or viruses of the influenza group exhibit this property. It is considered to be the result of the appearance of a new antigen on the cell surface brought about by receptor destruction (Burnet and Anderson, 1947).

By comparative titrations with normal and KIO_4-treated red-cells some irregularities of haemagglutination observed by various workers can be explained. Sheep-cells hitherto considered to be non-agglutinable under ordinary conditions by influenza viruses are rendered readily agglutinable by previous periodate treatment. Similarly treated fowl-cells give high agglutination titres with viruses of intermediate phases between "O" and "D", though not with true "O" viruses. If it is assumed that from normal cells the virus has eluted practically completely by the time the cells have settled, and this is the cause why the resulting pattern is that of "no agglutination", it will be clear that elution being impossible from KIO_4-treated receptors in this second case agglutination will be observed. This hypothesis is in complete agreement with the results of Briody (1948) who found that heated intermediate-phase viruses agglutinated fowl-cells, and with those of Stone (1948c) who observed similar high titre agglutination if the test was performed at 0° C. Clark and Nagler (1943) described the agglutination of sheep-cells by influenza viruses in the cold, and Gottschalk and Lind (1948) found that LEE virus heated previously for 30 minutes at 56° C. gave almost as high titres with sheep-cells as with fowl- or human-cells. As elution has been attributed to the virus-enzyme since the very first observation of the phenomenon by Hirst, it is only to be expected that either inactivation of the enzyme by heating or reduction of its activity by working at 0° C. should give final results similar to those produced by the modification of its substrate.

From the practical standpoint the use of KIO_4-treated fowl-cells furnishes a possible additional means of classifying O–D behaviour. Due to the high inihibitor content of amniotic fluids it is impracticable to perform agglutination tests in low dilutions of the reagents. In the case of an early ω-phase virus with doubtful fowl-cell agglutination, KIO_4-treated fowl-cells would be agglutinated to titres comparable with those given by human-cells, a fact which draws a sharp line between true "O" and intermediate forms.

The practical aspects of the reactions between PVR-cells and specific antibody will be reported in detail and discussed in a separate communication.

SUMMARY.

Metaperiodate modifies virus receptors; red-cells treated with suitable amounts of IO_4^- adsorb viruses of the influenza group, but neither spontaneous elution occurs, nor artificial removal by the "receptor-destroying enzyme" (RDE) of *V. cholerae*.

Erythrocytes treated with Periodate-Virus-RDE (PVR-cells) are stable in suspension and have active virus bound firmly to their modified receptors. PVR-cells have the following properties:

(a) They are not agglutinated by influenza viruses;

(b) They agglutinate all cells susceptible to influenza virus agglutinins;

(c) They destroy receptors on normal cells and the virus inhibitor in various mucoids;

(d) They are agglutinated by specific antisera to the virus with which they are coated;

(e) The agglutinating and enzymatic activities of these cells are neutralized by homologous antibody.

A quick method of preparing PVR-cells is given, and a new technique for antibody titration and antibody or inhibitor absorption is described.

The theoretical and practical significance of the results is discussed.

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MODIFICATION OF VIRUS RECEPTORS BY METAPERIODATE

II. INFECTION THROUGH MODIFIED RECEPTORS

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In the first paper of this series it has been shown that after the action of periodate in "modifying" doses, the receptors on the surface of red-cells are so altered that they retain the capacity of adsorbing influenza viruses, but neither spontaneous elution of the bound particle occurs, nor its artificial liberation by the "receptor-destroying enzyme" (RDE) of *V. cholerae*. It was concluded that by "modification" of receptors the substrate for the viral enzyme has been altered without impairment of the adsorptive capacity of the area. Thus enzymic activity is excluded from the phenomena of haemagglutination, rendering the virus-cell combination a practically irreversible one.

The hypothetical action of the viral enzyme has been postulated as a necessary preliminary to influenza infection (Hirst, 1942). As this assumption was based merely on the presence of an enzyme in the viral surface and the absence of any contradictory evidence, it was of interest to determine whether receptors could be modified *in vivo*, and if so, whether susceptible cells would be infected through modified receptors. It was believed that by this type of study some light might be thrown on the rôle of the enzyme in the initiation of infection, and at the same time on the share of the host cell in this process, an aspect hitherto unduly neglected.

MATERIALS AND METHODS.

All methods and materials used in the previous study (Fazekas de St.Groth, 1949) have already been fully described.

Horse serum saline. This was the diluent used in infectivity titrations, a sterile fluid containing 10 p.c. horse serum and 90 p.c. normal saline.

Ovomucin. The purified ovomucin (ovomucoid-β) was kindly provided by Dr. A. Gottschalk of this Institute. The preparation originally dissolved in 5 p.c. NaCl in distilled water was further diluted in normal saline. As high dilutions to give inhibitory titres of the order of hundreds were used in the experiments, the final NaCl concentration ranged between 0·90 and 0·95 p.c., only slightly above isotonicity.

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

Infectivity titration in eggs. The standard technique of Burnet (Beveridge and Burnet, 1946) was adopted.

Eleven-days-old white Leghorn eggs were given inocula of 0·05 ml., allantoically. The time of incubation was 42–48 hours at 35° C. The harvested fluids were tested for agglutinin by the routine technique, and the 50 p.c. infectivity end-points calculated by the formula of Reed and Muench.

Infectivity titration in mice. Healthy mice of uniform breed, obtained from the Institute's animal rooms, were used at the age of 6–8 weeks. Under light ether-chloroform (2:1) anaesthesia 0·05 ml. volumes of fluid were instilled intranasally. The lung lesions were evaluated at the time of death or at the end of the seventh day after the inoculation. Degrees of consolidation are designated as follows: 100 p.c. = 4, 75 p.c. = 3, 50 p.c. = 2, 25 p.c. = 1; specific death on the sixth day = 4·5, on the fifth = 5. The arithmetical mean is taken as the "average lung lesion" of a certain group.

The "excised lung technique". This method of assaying the number and state of available receptors in mouse lungs was developed in the course of two previous studies (Fazekas de St.Groth, 1948a, 1948b) and is fully described there.

EXPERIMENTS.

The Action of Metaperiodate on Influenza Viruses.

As in many of the subsequent experiments virus was exposed directly to the action of periodate, the effect of this chemical on the properties of active virus was investigated. All tests were performed with at least five different strains of the influenza group.

The Effect of Periodate on the Viral Agglutinin.

In the standard experiment 0·50 ml. virus-containing allantoic fluid and 0·50 ml. KIO_4 solution in normal saline were mixed and kept at room temperature for 30 minutes. KIO_4 was used in concentrations ranging from M/100 to M/100,000. After the end of treatment 0·10 ml. glycerol (0·1 M) saline was added to reduce the excess IO_4^- to IO_3^-. The samples were then tested by the routine haemagglutination technique for their agglutinin content. As controls virus treated with normal saline, and virus treated with the highest concentration of KIO_4 mixed with 0·10 ml. glycerol saline were included. Table 1 gives the readings of a typical experiment performed with the Melbourne strain of influenza A.

It can be seen that under the experimental conditions periodate has no effect whatever on the viral agglutinin. Identical results were obtained with the strains WS and IAN–D of influenza A, the LEE and Mil strains of influenza B, and Shope's strain "15" of swine influenza. Experiments with larger amounts of the chemical are detailed below.

The Effect of KIO_4 Treatment on the Phenomena of Adsorption and Elution.

Virus was treated with KIO_4 as in the experiment above. After the addition of glycerol saline the mixtures were kept in the refrigerator for 30 minutes. At the end of this time 0·50 ml. of the KIO_4-treated virus suspension and 0·50 ml. pre-chilled 1 p.c. fowl red cell suspension were mixed and kept at 4° C. During this "period of adsorption" virus combines with the cellular receptors, and—according to the result of control experiments—

TABLE 1.

The effect of periodate on the viral haemagglutinin.

(Melbourne A virus.)

Pre-treatment	Allantoic fluid + equal volume KIO_4 M/											Normal saline
	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	
Haemagglutinin titre	320	360	320	280	320	300	320	320	320	320	320	300

TABLE 2.

The effect of periodate on the viral enzyme.

(WS virus.)

Pre-treatment	Allantoic fluid + equal volume KIO_4 M/											Normal saline
	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	
Percentage of eluting agglutinin	0	16	44	100	100	100	100	100	100	100	100	100

TABLE 3.

The effect of periodate on the viral antigen.

(Virus of swine influenza.)

Pre-treatment	Allantoic fluid + equal volume KIO_4 M/											Normal saline
	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	
Titre of Standard antiserum	160	160	160	120	160	140	140	160	140	160	160	160

the equilibrium is practically reached in 15 minutes. After half an hour the tubes were centrifuged at low speed in the cold, and 0·05 ml. of the supernatant removed. The difference between the original titre of the used allantoic fluid and that of the sample removed represents the quantity of adsorbed virus. After sampling, the racks were shaken and kept in a water-bath at 37° C. for four hours. At the end of this period the tubes were centrifuged and an aliquot of the supernatant removed. The titre of this sample gives the quantity of eluted virus. (As seen in control experiments, spontaneous elution under the conditions of the experiment is complete in three hours). The results obtained with the classical WS strain of influenza A are given in Table 2.

Virus treated with an equal volume of M/100 KIO_4 has completely lost its capacity to elute. Even in the sample treated with M/400 only half of the virus comes off the red-cells spontaneously. The adsorbed agglutinin can be removed quantitatively by the use of the "receptor-destroying enzyme" of *V. cholerae*, thus showing that it is the enzymic activity of the virus which is impaired by the action of KIO_4.

Qualitatively all strains tested behaved similarly, though there were quantitative differences in the degree of resistance against KIO_4 treatment. The order is approximately LEE>BEL, WS, Swine>Melbourne, IAN-D, the LEE strain being the most resistant. The differences between the two extremes did not exceed fivefold.

The Effect of Periodate on the Antigenic Character of Virus.

This was tested in the following way: the virus-containing allantoic fluid was treated with KIO_4 as described above in the standard experiment, and diluted to contain 10 A.D. in 0·25 ml. Serial twofold dilutions of homologous antisera (ferret and rabbit) were made in normal saline and mixed with an equal amount of the virus suspensions. After 30 minutes at room temperature 0·25 ml. of 1 p.c. fowl cells (inhibitor insensitive) in normal saline was added, the racks shaken and the pattern of the settled cells read after 75 minutes' standing. Table 3 gives the results of an experiment with the virus of swine influenza.

As seen from the Table, KIO_4 treatment which did not alter the agglutinin content of the virus suspension, did not alter its antibody binding capacity either. Identical results were obtained by an alternative technique when KIO_4-treated virus was mixed with an excess of antibody, and the free antibody titrated back. To all appearances the periodate-treated virus behaved exactly as untreated virus did, with all strains tested. The *in vivo* test for antigenicity (capacity to elicit production of specific antibody) has not yet been done.

The Effect of Periodate on the Infectivity of Virus.

Infectivity titrations were made in eggs by the technique of Burnet. In a qualitative test 50–100 I.D. of virus were diluted in ten volumes of KIO_4. After 30 minutes at room temperature 0·05 ml. was inoculated by the allantoic route to each of six eggs. After an incubation of 42 hours at 35° C. the allantoic fluids were harvested and tested for agglutinin.

TABLE 4.

The effect of periodate on viral infectivity.

(Percentage of infected eggs.)

	Allantoic fluid + 10 volumes KIO_4						Controls	
	M/100	M/200	M/400	M/800	M/1,600	M/3,200	Normal saline	M/100 KIO_4 + glycerol
WS (A)	0	0	0	40	100	100	100	100
Mel (A)	0	0	0	17	83	100	100	100
BEL (A)	0	0	33	83	100	100	100	100
LEE (B)	0	0	17	66	100	100	100	100
Mil (B)	0	0	0	80	100	100	100	100
Swine	0	0	0	33	100	100	100	100

Again, there is some variation among the strains tested, the B strains being somewhat more resistant. It is clearly borne out by the controls that the effectiveness of KIO_4 is nullified by previous reduction with glycerol saline.

The Influence of Temperature, Concentration and Time of Action on the Effects of Periodate Treatment on Virus.

A series of the above experiments (with LEE virus) was repeated at 0°, 20°, and 37° C., with KIO_4 treatments lasting for 5–10–15–30 minutes. It was found that at room temperature and at 37° C. there was no difference between the effect of KIO_4 after 5 or 30 minutes, while at 0° C. it took $>10 <15$ minutes to obtain similar results. These data are not sufficient to compute the temperature coefficient of the interaction between periodate and virus, but to all appearance it is a reaction with a Q_{10} less than 2, i.e. of the order of an ordinary chemical reaction.

To test the effect of concentration and relative quantities of the reagents the following set-up was used:

A standard amount of virus (0·25 ml. allantoic fluid) was treated with standard amounts of KIO_4 diluted to give final concentrations from M/100 to M/10,000. Thus the concentration of the reagents and their relative quantities were varied independently. After 60 minutes at room temperature KIO_4 was neutralized by twice the sufficient amount of glycerol saline and the sample tested for agglutination, adsorption-elution and antigenicity. A summary of the results is given in Table 5.

Extensive periodate treatment destroys not only the eluting capacity of the virus, but its power to agglutinate and bind antibody as well. The two latter properties—as far as can be judged from the results—disappear *pari passu*. By the comparison of the relative amounts of KIO_4 necessary to produce these changes it is evident that this is not a "concentration effect", but there exists a stoichiometric relationship between the quantity of periodate and the change produced. A suitable amount of the chemical in a concentration M/1,000 produces a change in the behaviour of the virus which half the quantity, though applied in a concentration M/100, would not produce.

TABLE 5.

The effect of concentration on the results of periodate action.

(LEE virus.)

Relative amount of KIO_4 (mg./ml. of allantoic fluid)	Haemagglutination						Antigenicity						Enzyme action					
	Final concentration of KIO_4 M/																	
	100	200	400	800	1,600	3,200	100	200	400	800	1,600	3,200	100	200	400	800	1,600	3,200
16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	tr	tr	±	±	±	±	—	—	—	±	±	±	—	—	—	—	—	—
4	++	++	++	++	++	++	++	++	++	++	++	++	—	—	—	tr	tr	±
2	++	++	++	++	++	++	++	++	++	++	++	++	tr	—	—	±	±	+
1	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	±	+	+
0·5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = reactions of untreated virus; ++ = increased reaction; ± = decreased reaction; — = no reaction.

In spite of the fact that constant proportions of the reagents are found to be involved in the interaction, it is impossible to determine the quantity of KIO_4 that would inactivate virus, as the allantoic fluid itself reduces periodate by virtue of its carbohydrate and protein content; similarly, it is probably not only the essential constituents of the infective particle which are acted upon by IO_4^-, but also the inert parts of the virus (the "normal component" of Knight).

The Properties of Virus Partially Inactivated by Periodate.

It is known that most influenza B strains when heated to 56° C. for 30 minutes (Francis, 1947) or acted upon by trypsin for a suitable period (Stone, 1948) lose their receptor-destroying activity and at the same time become detectors for inhibitor. In the case of heat inactivation there is a close correlation between the loss of enzyme activity and the degree of inhibition by mucoids (Burnet, 1948a). As certain amounts of KIO_4 inactivated the viral enzyme without destroying the agglutinin, it was of interest to test whether virus treated in this fashion would be inhibited by inhibitors for heated B viruses.

Periodate-treated virus suspensions which had no enzyme activity when tested against 100 inhibiting doses of ovomucin, were diluted to contain 5 A.D. in 0·25 ml., and mixed with an equal volume of an ovomucin dilution in normal saline. After 30 minutes at room temperature 0·25 ml. of 1 p.c. "inhibitor sensitive" fowl-cells was added and the test read after 75 minutes. As controls titrations of the same ovomucin with 5 A.D. of active and heated LEE virus were included.

TABLE 6.

Inhibitor sensitivity of LEE virus after periodate treatment.

Pre-treatment of virus	Dilution of ovomucin							Titre
	1:10	1:30	1:90	1:270	1:810	1:2,430	1:7,290	
1·0 mg. KIO_4 / ml. all. fluid	++	++	++	++	++	++	++	<10
2·5 mg. KIO_4 / ml. all. fluid	—	tr	+	++	++	++	++	90
5·0 mg. KIO_4 / ml. all. fluid	—	—	—	--	±±	++	++	600
10 mg. KIO_4 / ml. all. fluid	++	++	++	++	++	++	++	<10
30 min. 56° C. ("heated LEE")	—	—	—	—	—	tr	±±	5,000
Nil ("active LEE")	++	++	++	++	++	++	++	<10

The results show that the LEE virus becomes an indicator for inhibitor if its enzymatic activity has been destroyed by KIO_4 treatment. However, there is a marked quantitative difference between the resultant of heat and periodate

inactivation, the former being 10 times more sensitive to inhibition. The significance of this finding will be discussed below.

These findings bear out that periodate has no effect on influenza viruses in quantities to be used in the experiments following below. Furthermore, it is evident that glycerol saline nullifies the effectiveness of IO_4^- by reducing it to IO_3^-. This technique of neutralization was used in all tests.

THE EFFECT OF PERIODATE ON RECEPTORS OF THE RESPIRATORY TRACT.

Preliminary Experiments on Excised Lungs.

The respiratory tract of the lung preparations was rinsed with three changes of normal saline prior to all experiments. Periodate treatment consisted in aspirating 0·5 ml. volumes of suitable dilutions and keeping them in the lungs for a period of ten minutes. The chemical was used in concentrations ranging from M/100 to M/25,000. After the removal of periodate the lungs were washed with three changes of glycerol (0·1 M) saline, and then a standard dose (1,000 A.D.) of active LEE virus was run into the lungs and kept there for 10 minutes. Following the virus treatment the respiratory tract was washed every ten minutes during the first, three times in the second and twice in the third hour with 0·5 ml. volumes of normal saline. Finally 0·5 ml. RDE in calcium saline (titre 400) was added and removed after 15 minutes' contact with the bronchial surface. All washing fluids following the administration of virus were tested for their agglutinin content. The results (average of eight experiments) are given in Table 7.

TABLE 7.

Receptor modification by periodate in excised lungs.

(Reaction of untreated lungs taken as 100 p.c.)

	Pre-treatment of lungs: KIO_4 M/									Control
	100	200	400	800	1,600	3,200	6,400	12,800	25,600	Normal saline
"adsorption"	55	70	100	100	100	100	100	100	100	100
"spontaneous elution"	0	0	0	0	0	0	8	30	60	75
"artificial liberation"	0	0	0	0	0	2	15	60	80	100

There is a striking similarity between the results obtained in excised lungs and with red-cells (see previous paper). Below a certain concentration no spontaneous elution of virus occurs, and RDE is incapable of removing the bound agglutinin. It is clearly borne out by this experiment that respiratory receptors are modified by periodate in the same manner as are those of red-cells.

To test whether the process of receptor modification would take place *in vivo*, the following experiment was performed.

Groups of mice were given 0·05 ml. volumes of KIO_4 in normal saline intranasally. The animals were sacrificed one hour later and the excised lungs prepared for the adsorption-

elution experiment. The respiratory tract was washed with three changes of glycerol (0·1 M) saline before adding the standard amount of LEE virus. From then on the experiment was the same as the one described in the previous paragraph. The results are given in Table 8.

TABLE 8.

Receptor modification by periodate in living mice.
(Reaction of saline-treated lungs taken as 100 p.c.)

	Pre-treatment: intranasal instillation KIO_4				Normal saline
	M/100	M/200	M/400	M/800	
"adsorption"	100	100	100	100	100
"spontaneous elution"	5	25	70	80	75
"artificial liberation"	12	45	85	100	100

It is clear that the virus receptors of the respiratory tract are modified *in vivo* as well as *in vitro*. However, there is a quantitative difference, as the degree of modification after 0·05 ml. KIO_4 M/100 corresponds to the effect of 0·5 ml. KIO_4 M/6,000 in the excised lung, i.e. only one-sixth of what could be expected.

This is in complete agreement with the finding made in the course of a previous study on the effect of intranasal RDE instillation (Fazekas de St.Groth, 1948b), where only 15–20 p.c. of the theoretical result could be observed. An explanation for the phenomenon is given in the quoted paper.

Similar experiments were performed on mice killed at longer intervals after the instillation of KIO_4. In sharp contrast to the rapid regeneration of receptors after destruction by RDE, the modified receptors persisted for a longer period (at least four days), and reversal to the normal state took at least two weeks. A comparative study of the regeneration and restitution of receptors will be given in a later paper of this series.

Infection of Mice through Modified Receptors.

In the previous section it has been shown that by intranasal administration of periodate respiratory virus receptors can be modified *in vivo*. By this means the possibility was given of testing the viral infectivity through receptors on which the enzyme of the infective particle is incapable of action.

It is known from the studies of Stone (1947, 1948a, 1948b) that the "receptor-destroying enzyme" of *V. cholerae* is a potent prophylactic against influenza infection by virtue of its activity on the host-cell. In the first paper of this series it has been shown that neither RDE nor the viral enzyme is capable of destroying receptors modified by KIO_4. Consequently, if enzymic receptor destruction is essential for the entry of the infective particle into the host cell, no infection should take place through modified receptors, and

RDE should be more effective in preventing infection as it removes the virus from normal receptors, the only ones through which it might get into the cell, and whose number—due to KIO_4 treatment—is relatively low. If, on the other hand, enzymic destruction of receptors is not a prerequisite of infection, a standard amount of virus should give identical infectivity end-points both in normal and periodate-treated lungs, further, RDE should be less effective as a prophylactic agent, as it is incapable of removing virus adsorbed to KIO_4-modified receptors.

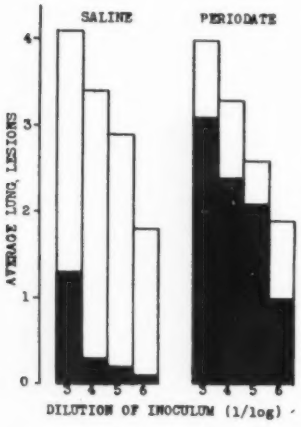

Fig. 1. *Infection of periodate-treated mouse lungs.* (Melbourne A virus.)

□ virus inoculum in saline.

■ virus inoculum in RDE.

Mice were given 0·05 ml. KIO_4 M/100 intranasally under light anaesthesia and after an hour's interval a second inoculum of 0·05 ml. containing dilutions of Melbourne virus in either horse serum (10 p.c.) saline or in a concentrated RDE preparation (titre 6,000). Control mice were pre-treated with normal saline instead of KIO_4, and received after one hour the same inocula as the periodate-treated ones. This part of the experiment was essentially a repetition of Stone's experiment on the prophylactic value of RDE (1948b). Mice instilled intranasally with RDE or KIO_4, and a combination of these two were included as negative controls. Groups of six mice were used for each dilution, and the lung lesions evaluated at the time of death or at the end of the seventh day. Fig. 1 shows the results.

In the control group—as a perfect confirmation of Stone's findings—RDE reduced the specific lung lesions by more than thousandfold (1:1,330). In the group of mice pre-treated with KIO_4 the lung lesions caused by equal amounts of virus are the same as in the control mice. The protective effect of RDE on the other hand was more than hundred times less in this group (1:12). This difference is statistically significant with a probability of 97/100. Thus it is concluded that—as RDE is incapable of removing virus from KIO_4-treated receptors—it is the modified receptors through which infection has been initiated.

The Effect of Periodate on Receptors of the Allantoic Cavity.

Preliminary Experiments.

In the first series of experiments the allantoic cavity was inoculated with 0·10 ml. 4 p.c. formaldehyde, by which treatment the embryo is killed in less than one hour and the cells lining the cavity in less than 20 minutes. An hour later the eggs were given an inoculum of KIO_4 M/100 in normal saline, in volumes ranging from 0·25 to 1·0 ml. Controls received the same volumes of saline. The eggs were then placed for half an hour in the refrigerator at 4° C., at the end of which period they were given 0·10 ml. of an active LEE virus preparation (titre 800). After another 30 minutes at 4° C., during which time adsorption of the virus to the allantoic lining was practically complete, a sample of 0·25 ml. was removed (a), and the eggs put in an incubator at 35° C. for two hours. A second

sample (b) of 0·25 ml. was then taken and replaced by an equal amount of RDE, titre 1,500. After 30 minutes in the incubator a third sample (c) was removed. The three samples were tested for their agglutinin content, dilutions starting 1:2. Fig. 2 gives the results. Each point represents the average obtained from 36 eggs. As the variation in the quantity of allantoic fluid is more than ± 0·5 ml., no correction has been taken for the reduction in volume due to sampling, the Figure gives the values of free agglutinin as read in the tests.

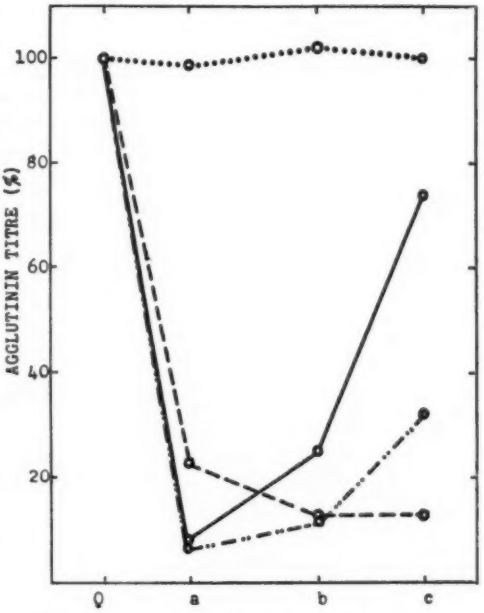

Fig. 2. *Adsorption-elution of virus in formolized allantoic cavities.* (LEE virus.) Pre-treatment of eggs:

———— normal saline (1·0 ml.).
•••••••••• RDE (0·5 ml.).
- - - - - - - - - IO_4^- M/100 (1·0 ml.).
—•·—•·— IO_4^- M/100 (0·5 ml.).

"a" = titre after 30 minutes at 4° C. (adsorption).
"b" = titre after 120 minutes at 35° C. (spontaneous elution).
"c" = titre after the addition of RDE (artificial liberation).

Again, receptors were modified by suitable amounts of KIO_4: they adsorbed virus, but neither spontaneous elution nor artificial liberation by RDE occurred. The amount of periodate necessary to produce this change is unusually high. However, it has to be borne in mind that allantoic fluids reduce periodate, so that only a fraction of the amount inoculated reaches and modifies receptors. This was clearly shown by some control experiments in which the original allantoic fluid was removed and replaced by saline before the experiment. In this case roughly less than one-tenth the amount (0·5 ml. M/600) produced the same effect as 1·0 ml. M/100 in the above experiment.

In spite of this finding, the allantoic fluids were not exchanged for saline in the subsequent experiments on living eggs, as this procedure would have immensely increased the probability of contamination.

Infection of Eggs through Modified Receptors.

To test whether periodate treatment influences the end-point of an infectivity titration, groups of six eggs were inoculated with 0·05 ml. horse serum saline containing falling tenfold dilutions of virus. One lot of eggs was given 1·0 ml. KIO_4 M/100 30 minutes prior to the virus inoculum; controls received the same volume of normal saline. The

results are tabulated below; infectivity end-points were calculated according to Reed and Muench.

TABLE 9.

Infectivity end-points in normal and periodated eggs.

(The titres are the negative logarithms of the dilution giving 50 p.c. infection.)

Pre-treatment	Virus strain							
	WS	Mel	Cam	IAN	Bel	LEE	Mil	Swine
Saline	8·0	8·1	6·3	5·5	8·0	8·0	6·7	7·0
Periodate	8·3	7·9	6·1	5·3	7·7	8·0	7·2	6·5

As in mice, the infectivity end-points in normal and KIO_4 pre-treated eggs were identical with all virus strains tested.

In a second experiment, to test whether the number of available receptors was reduced by periodate treatment, a method based on the principle of interference was used. It is known that inactive virus interferes with the multiplication of active virus. A mixture of active and inactive particles yields larger amounts of virus in the allantoic cavity when inoculated in high dilutions than in the undiluted state. It is assumed that the inactive particle blocks the entry of the active virus into the future host cell. If this is so, then the same result should be obtained by reducing the number of available cells as by increasing the concentration of the virus mixture.

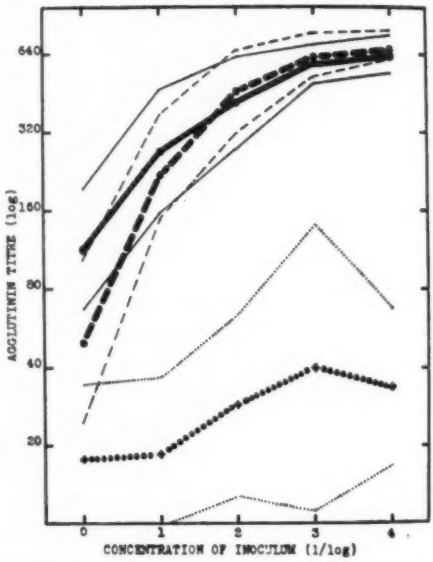

Fig. 3. *Interference curves of active and heated LEE virus in allantoic cavities.* Pre-treatment of eggs:

——— KIO_4.
- - - - - - - - - Saline.
••••••••• RDE.

Heavy lines represent the average, light lines the ± standard deviations.

In the actual experiment a mixture of 100 parts of heat-inactivated (30 minutes at 56° C.) and one part of active LEE virus was used as inoculum. Groups of eggs were pre-treated with KIO_4, normal saline or RDE, and after incubation for half an hour at 35° C. the reagents were neutralized by an inoculum of 0·10 ml. glycerol saline containing anti-RDE serum. Finally, 0·10 ml. of the "interfering virus mixture" was given allantoically in tenfold dilutions, ranging from 10^0 to 10^{-5}. After incuba-

tion for 42 hours the allantoic fluids were harvested and tested for their agglutinin content. Fig. 3 gives the results.

The "interference curves" of normal and KIO_4-treated eggs are identical, whereas eggs treated with RDE—an agent known to destroy receptors—give the typical picture which should be expected if the number of available receptors would be reduced. From this experiment it is concluded that the amount of periodate which modifies receptors, does not reduce the number of receptors available for infection. This at the same time is an indirect proof that infection takes place through modified receptors.

In a third experiment—based both on the phenomenon of viral interference and the infection-reducing effect of RDE—a direct proof for the same point was furnished.

In a previous study on the adsorptive affinities of different influenza virus strains to cellular receptors (Fazekas de St.Groth, 1949, in preparation) it was found that the LEE strain, either active or heat-inactivated, was more readily adsorbed to susceptible cells than, e.g. the Melbourne strain. Thus in a competition for receptors, especially if the number of LEE particles in the system markedly exceeds that of the Melbourne virus, one would expect all receptors to be blocked by the former.

In the actual experiment eggs were pre-treated with 1·0 ml. of KIO_4 M/100 or the same volume of normal saline, and inoculated half an hour later with 0·05 ml. active Melbourne virus diluted 1:10,000 in horse serum saline. After an incubation period of 30 minutes at 35° C., during which time the virus is adsorbed to receptors but is still removable by RDE, a third inoculum of 0·5 ml. was given. This contained four parts of RDE and one part of heated LEE virus. The amount of LEE in the allantoic cavity, as judged by the agglutinin titres, was 15,000 times more than that of Melbourne virus. As controls groups of eggs were given 0·10 ml. of heated LEE virus without RDE as a third inoculum.

TABLE 10.

Infection of eggs through modified receptors.

(for description see text.)

Average agglutinin titres.

Pre-treatment	Second inoculum		
	Nil	Heated LEE	Heated LEE + RDE
Saline	1,200	280	0
Periodate	1,200	246	102

It was expected that in the saline pre-treated eggs RDE should remove the Melbourne virus from the adsorbed state and thus initiate a true competition between the active and inactive particles for the small number of available receptors. This competition should turn out in favour of the LEE virus, due to its higher adsorptive affinity and large excess, and the eggs should not be infected. In the periodate-treated eggs on the other hand there is no possibility of true competition as RDE is incapable of removing the Melbourne virus

already adsorbed to modified receptors. Thus these eggs should be infected in spite of the presence of RDE and a vast excess of interfering inactive virus in the allantoic cavity.

As seen from Table 10, the prediction was fully verified by the experiment. In saline pre-treated eggs the yield of virus was significantly reduced by the presence of interfering inactive LEE virus, though all eggs were infected. In the periodate-treated eggs the effect of the straight virus inoculum, and virus followed half an hour later by an interfering dose, was identical with the controls. There is, however, a striking difference in the results of the groups receiving RDE. All periodated eggs *are* infected, but none of the controls. The average agglutinin titre of these allantoic fluids is somewhat lower than in the group which did not receive RDE; this can be attributed to the destruction of RDE-sensitive receptors formed during the period of incubation.

DISCUSSION.

The present study was initiated to determine whether the action of IO_4^- on cells susceptible to influenza infection was essentially similar to its action on red-cells. The results show clearly that periodate does in fact modify receptors in the respiratory tract of mice and in the allantoic cavity of developing eggs. Furthermore, this reaction takes place not only on non-living substrates but also *in vivo*, apparently without impairing the viability of the respective experimental animal. As on red-cells, receptors modified in the living state adsorb virus, but neither spontaneous elution occurs, nor artificial liberation by the "receptor-destroying enzyme" (RDE) of *V. cholerae*. As there is an absolute agreement between results obtained on living and non-living systems, it is concluded that the action of periodate is the same in both cases: the substrate for the viral enzyme or RDE is altered, and thus the activity of these enzymes rendered impossible. In favour of this assumption is the finding (to be reported in a later communication) that RDE fails to combine with sufficiently periodated red-cells which still bind influenza viruses.

In preliminary tests it was found that by "modifying doses" of IO_4^- none of the known properties of the infective particle was altered, and that the action of the chemical could be completely neutralized by glycerol saline. Thus it was safe to adapt the method to *in vivo* infectivity experiments.

It has been shown by four independent methods, both in mice and chick embryos, that cells can be infected through modified receptors on which viral enzyme is incapable of action.

This necessitates a considerable modification of Hirst's original conception of the process of infection by influenza virus (1942), a conception that was also tacitly adopted in earlier papers from this laboratory. According to this hypothesis the virus through its enzyme component unites with and then

destroys the cellular receptor, the complete process being a prerequisite for infection. The experiments reported in this paper show that infection can be initiated by union of virus to receptors which have been rendered quite resistant to the action of viral enzyme and RDE. It should also be realized that enzyme-like action was hitherto demonstrated *only* on non-living or non-host cells (excised lungs, formolized allantoic linings, red blood corpuscles), and was invariably followed not by the entrance of the virus into, but its separation from these cells. No positive results have been obtained with living, susceptible cells.

The simplest interpretation of the facts is that the virus adsorbed to the surface of the susceptible cell enters the cytoplasm by a passive process, analogous to the phenomenon known as colloidopexis ("Speicherung", athrocytosis), the uptake by cells of submicroscopical particles.

On such a view the process of infection is initiated by physical combination between two surfaces of complementary pattern, the "receptor area" of the cell and the viral "combining area" ("B spots" in the terminology of Burnet, 1934), followed by *"viropexis"*. The fact that such a union will under normal circumstances probably result in partial or complete destruction of the receptor is irrelevant. Further support for this view is the finding that heat-killed virus lacking both enzymic and "infective" (multiplying) activities is taken up by the cells of the allantoic lining as readily as active virus. These experiments are published elsewhere (Fazekas de St.Groth, 1948c).

One more point calls for some discussion. In the preliminary tests it was found that LEE virus treated with appropriate amounts of periodate loses its enzyme activity without loss of its agglutinating power (actually the agglutinin content is increased, similarly as in experiments of the Henles (1947) after ultraviolet irradiation, or in those of Stone (1949) after mild tryptic digestion). Periodate-inactivated virus has thus the same properties as heat-inactivated virus, and should serve as a detector for inhibitors. This in fact is the experimental finding, though there is a marked quantitative difference if an inhibitor is assayed by the two types of inactive virus. The loss of enzyme activity runs parallel with the development of inhibitor sensitivity, as found by Burnet (1948a, b), but the final level of sensitivity depends on the mode of inactivation.

SUMMARY.

By treatment with relatively large amounts of periodate the infectivity, enzyme activity, antigenicity and haemagglutinin of influenza viruses can be destroyed. Reduction of IO_4^- to IO_3^- nullifies its effectiveness.

Cellular receptors in the excised mouse lung and in formolized allantoic cavities can be modified by periodate: they adsorb virus, but neither spontaneous elution nor artificial liberation by the "receptor-destroying enzyme" (RDE) of *V. cholerae* occurs.

Identical receptor modification *in vivo* is produced, both in mice and eggs, without any adverse effect on the host.

It is shown by four methods that influenza infection may take place through modified receptors on which receptor-destroying enzymes cannot act:

Identity of infectivity end-points in periodated and normal mice and eggs.

Identity of "interference curves" in eggs.

Hundredfold reduction of the prophylactic effect of RDE in periodated lungs.

Absence of interference in the allantoic cavity by heat-inactivated virus in the presence of RDE, if the active virus has been adsorbed to receptors modified by periodate.

The assumption of enzymic receptor destruction as the mechanism of infection (Hirst) is criticized, and a new hypothesis proposed: "Preceded by specific adsorption of the virus to cellular receptors, infection is initiated by means of *viropexis*, the passive uptake of the infective particle by the host-cell; enzymic destruction of receptors is not essential."

Acknowledgment. It is a pleasure to thank Professor F. M. Burnet for his interest and inspiring criticism.

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THE TOTAL PHOSPHORUS, PHYTATE PHOSPHORUS AND INORGANIC PHOSPHORUS OF BREAD AND THE DESTRUCTION OF PHYTIC ACID IN BREAD MAKING

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Evidence has been presented by Hoff-Jorgensen *et al.* (1946), Mollgaard *et al.* (1946) and the Cereals Committee, Copenhagen (1946) that, under some conditions, phytic acid is not available as a source of phosphorus and interferes with calcium absorption. On the other hand Cruickshank *et al.* (1945) have shown that the phytic acid of oatmeal disappears entirely from the ingesta during its passage through the intestine, and Spitzer *et al.* (1948) that rats can effectively utilise phytate phosphorus in the form of calcium phytate, provided that there is an adequate intake of vitamin D.

Data on the phytate phosphorus content of Western Australian white flour have been presented (Lee and Underwood, 1948), although from the standpoint of human nutrition the phytate content of bread is more important than that of the actual cereal or its flour, since cereals are consumed largely in this form. Di Stefano and Muntoni (1938–9) and Pringle and Moran (1942) have shown that the destruction of phytic acid during the dough preparation for bread baking is considerable, due to the presence of phytase, and that no further destruction occurs on baking. The degree of this destruction depends on a number of factors, of which the most important seem to be the amount of phytate originally in the flour and hydrogen ion concentration of the dough. No data on the phytic acid phosphorus content of Australian bread or the extent of phytic acid destruction in Australian bakeries appear to have been published, prior to the results presented in this paper, apart from a few figures given in the Annual Report of the Government Analyst, Queensland (1946).

EXPERIMENTAL.

Sampling.

Two methods of sampling were used.

(i) A preliminary survey of the phytate phosphorus content of bread consumed in the metropolitan area of Perth was carried out by collecting five samples of bread from widely-separated vendors.

(ii) A second series of samples was collected in such manner as to give a more accurate measure of the percentage destruction of phytic acid in bread making. Flour (or "whole-

[1] Working under a grant from the National Health and Medical Research Council.

meal'') was sampled just prior to dough mixing in each of six bakeries, and the bread sample collected was made from this flour or ''wholemeal''. Six white flour—white flour bread samples and six ''wholemeal''—''wholemeal'' bread samples were collected in this way. The ''wholemeal'' breads were baked from meals containing various percentages of whole wheatmeal mixed with white flour; in only one of the bakeries was a 100 p.c. whole wheatmeal bread baked.

Two pound ''Devon'' loaves were used in all cases. The whole loaves were cut in slices about 2 c.m. thick, oven dried at 70°–80° C. and then ground through a 30 mesh sieve prior to analysis.

Methods of Analysis.

The methods for total phosphorus, phytate phosphorus and inorganic phosphorus have been described previously (Lee and Underwood *loc. cit.*).

Where the phytate phosphorus content of the bread to be analysed was less than 0·01 p.c. it was found more convenient to introduce a small modification. Phytin was prepared by the method of Boutwell (1917). A solution was prepared containing 1·1 gm. of this salt in one litre of N/3 hydrochloric acid. 10 gm. of bread were shaken with 100 ml. of N/3 hydrochloric acid containing 10 p.c. sodium sulphate. After filtering, a 15 ml. aliquot of this extract was treated in a centrifuge tube with 5 ml. of the phytin solution and 5 ml. of ferric chloride solution (0·6 p.c. Fe+++). In a second tube 15 ml. of hydrochloric acid-sodium sulphate extracting solution were treated in the same way. The rest of the procedure was as described by McCance and Widdowson (1935). The difference in the phosphorus content of the two 15 ml. aliquots (extract and extracting solution) is due to the phytate phosphorus of the bread.

RESULTS.

The results of analyses of white flour and bread, collected by sampling method (ii) are given in Table 1. The total phosphorus, phytate phosphorus and inorganic phosphorus contents are expressed as mean percentages of dry weight together with the coefficients of variation. The percentage destruction given in this Table is calculated directly from the results of the flour and bread phytate analyses.

TABLE 1.

Total, phytate and inorganic phosphorus of white flour and bread. Percentages on dry basis.

Material	Total phosphorus		Phytate phosphorus		Inorganic phosphorus		Percentage destruction of phytate	
	Mean percentage	Coefficient of variation	Mean percentage	Coefficient of variation	Mean percentage	Coefficient of variation	Mean	Coefficient of variation
Flour	0·085 ±0·002	7·8	0·026 ±0·001	13·9	0·004	2·7		—
Bread	0·088 ±0·006	15·9	0·007 ±0·002	54	0·032 ±0·005	37	72 ±3	10·7

Table 2 gives the results of all white bread analyses (11 samples) for total and phytate phosphorus. Where the bread was collected by the first method of sampling the approximate percentage destruction of phytic acid was calculated by assuming the original phytate phosphorus content of the flour to be 29·4 p.c. of the total phosphorus of the bread. The figure 29·4 p.c. is the average percentage total phosphorus occurring as phytate in flour (Lee and Underwood, 1948).

TABLE 2.

Total and phytate phosphorus of white bread (all samples). Percentages on dry basis.

Material	Total phosphorus		Phytate phosphorus		Percentage destruction of phytate	
	Mean percentage	Coefficient of variation	Mean percentage	Coefficient of variation	Mean	Coefficient of variation
Bread	0·092 ±0·004	12·0	0·007 ±0·001	41	74 ±3	8·4

The total phosphorus, phytate phosphorus and inorganic phosphorus contents of the six "wholemeal" flours and the corresponding "wholemeal" breads, together with the approximate percentage destruction of phytic acid, are given in Table 3.

TABLE 3.

Total, phytate and inorganic phosphorus of "wholemeal" and "wholemeal bread". Percentages on dry basis.

Material	Total phosphorus	Phytate phosphorus	Inorganic phosphorus	Percentage destruction of phytate
Meal	0·151	0·095	0·008	62
Bread	0·149	0·036	0·072	
Meal	0·251	0·157	0·015	59
Bread	0·254	0·065	0·13	
Meal	0·126	0·067	0·007	56
Bread	0·126	0·029	0·046	
Meal	0·149	0·073	0·007	60
Bread	0·160	0·029	0·065	
Meal	0·121	0·061	0·006	54
Bread	0·159	0·028	0·083	
Meal	0·134	0·069	0·006	78
Bread	0·120	0·015	0·047	

Two white flour breads and two "wholemeal" breads had phosphorus in the form of calcium phosphate added before baking. This would account for the higher total phosphorus content of the bread as compared with the total phosphorus of the flour.

There was considerable variation between bakeries in the extent of the destruction of phytic acid but neither the dough time nor the percentage yeast appeared to alter this destruction significantly. In all bakeries compressed yeast was used in amounts ranging from 0·33 p.c. to 0·75 p.c. The "dough time" varied from 7–10 hours with the different bakeries.

DISCUSSION.

The figures obtained in the present investigation for the total, phytate and inorganic phosphorus contents of white flour are practically identical with those previously presented by the authors and show little individual variation. The total phosphorus of the white breads corresponds very closely with that of the flours from which they were made, the slightly higher mean figure being due to the fact that in two cases calcium phosphate was added to the flour before baking. The total phosphorus contents of the "wholemeal" flours and breads are much more variable, due to the varying proportions of white flour mixed with the whole wheatmeal. The figures indicate that, with one exception, these so-called "wholemeals" contain at least 50 p.c. white flour.

A feature of the results is the very much greater variability in the phytate phosphorus contents of the breads than of the flours or meals from which they are made. This high variability applies also to the results for the percentage destruction of phytic acid. This destruction is appreciably greater, on the average, in the white breads than in the breads made from higher extraction flour, but a more significant variation seems to be between bakeries. A variation between bakeries is not surprising in view of the finding of Pringle and Moran (1942) that reducing the pH of "National Wheatmeal" dough from the normal figure of 5·6–5·7 to 5·44 and 5·21 increased the destruction of phytic acid from 58 to 64 p.c. to 91 and 99 p.c. respectively. In local bakeries the pH of dough is not controlled, so that small differences in the methods of preparation might vary considerably the extent of phytic acid destruction.

The phytate phosphorus figures for white bread given in Tables 1 and 2 are so small that phytic acid consumed from this source can hardly interfere with calcium absorption to a significant extent. The position with respect to the "wholemeal" breads can, however, be quite different, depending upon the degree of extraction of the flour. For instance, the consumption of 1 lb. of a bread containing 0·065 p.c. phytate phosphorus, the highest of the figures given in Table 3, will theoretically immobilize 0·4 gm.–0·6 gm. of calcium, if the ratio given by Pedersen (1940) is accepted. This author states that 1 part of calcium is precipitated by 1·1–1·6 parts of phytic acid. This is an extremely large amount of calcium, representing a very high proportion of the normal daily intake. It must be viewed with reservation in view of the many dietary

factors such as protein, fat and vitamin D known to influence calcium absorption, even in the presence of phytic acid. Moreover, it represents a probable maximum figure from the consumption of almost 100 p.c. wholemeal bread. Very small amounts indeed of such bread are consumed in Australia and only very limited amounts of bread made from the more favoured 70:30 or 60:40 mixtures of wholemeal and white flours. Such bread would, by calculation from the figures for wheat and white flour given in the previous paper (Lee and Underwood, 1948), and assuming a phytate destruction of 65 p.c., contain on the average 0·045 and 0·040 p.c. phytic acid phosphorus respectively. Consumption of a 1 lb. loaf of such bread would, using again the ratio given by Pedersen (1940), precipitate 0·25 gm.–0·4 gm. calcium as insoluble calcium phytate. Although as just pointed out, very little bread of this sort is consumed in Australia as a whole, these figures may be of particular importance for those individuals or families who for a variety of reasons consume the whole of their bread in this form.

It should be emphasized that, although these calculations show clearly the difference between white and "wholemeal" breads in respect to phytic acid content and their potential effects on calcium absorption, they tell nothing of their actual effects under various dietary regimes. Much further study of this problem, including long-term Ca balance experiments with humans, under defined conditons, is necessary before the results can be successfully interpreted in this respect. For instance, Walker, Irving and Fox (1946) found that high phytate diets did not induce *permanent* negative calcium balance in humans.

The inorganic phosphorus figures given in Table 1 show that the increase in the bread, compared with the meal from which it is made, is rather greater than the decrease in phytate phosphorus. Apparently other phosphorus compounds, such as phospholipids or nucleo-proteins, are also hydrolysed during bread-making.

SUMMARY.

Samples of white bread and "wholemeal" bread, and of the flours from which they were made, were collected from six bakeries and analysed for total, phytate and inorganic phosphorus.

The white flours averaged 0·085 p.c. total, 0·026 p.c. phytate, and 0·004 p.c. inorganic phosphorus on the dry basis and showed little individual variation. The corresponding figures for the breads made from these flours were 0·088, 0·007 and 0·032 p.c. The mean percentage destruction of phytic acid was 72 p.c.

The "wholemeal" flours and breads were very much more variable in composition and evidently contained widely varying proportions of white flour mixed with whole wheatmeal. In all cases they were much richer in total phytate phosphorus than the white flour or breads. The destruction of phytic acid ranged from 54 to 78 p.c.

The variation in destruction of phytic acid was related more to the bakeries than to the phytate content of the flour. Differences in hydrogen ion concentration of the dough are considered to be the cause of this variation between bakeries.

The marked difference between white and "wholemeal" breads in phytic acid content is stressed and the possible significance of this fact in relation to calcium absorption is discussed.

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OESTROGENIC EFFECTS OF SUBTERRANEAN CLOVER (*T. SUBTERRANEUM* L. VAR. DWALGANUP): PROTECTIVE ACTION OF ANDROGEN IN THE CASTRATE MALE[1]

by JUNE EAST, E. J. UNDERWOOD AND H. W. BENNETTS

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Bennetts, Underwood and Shier (1946) have described a breeding problem in ewes maintained on pastures in which the Dwalganup strain of subterranean clover is the principal plant species. The disease is manifested clinically by female infertility, maternal dystocia and prolapse of the uterus, and pathologically by the occurrence of cystic changes in the endometrium and other abnormalities in the uterus. Castrated male (wether) sheep maintained on these pastures have been shown by Bennetts (1946, 1947) to exhibit squamous metaplasia of the secondary sex organs, particularly of the bulbo-urethral glands. These conditions were attributed to the presence in the clover of an oestrogenic substance or its precursor. Later work (Curnow, Robinson and Underwood, 1948) demonstrated the presence of such a substance in ether extracts of samples of subterranean clover from "affected" properties. The feeding of these extracts to mice and guinea-pigs produced uterine and vaginal changes similar to those induced by the injection of oestradiol.

A feature of the breeding problem is that entire male sheep are quite unaffected by the "potent" clover. In explanation of this fact it was argued that the androgen produced by the animal's testicles was adequate to protect it against the effects of the oestrogenic substance ingested from the clover. Prior to the experiments reported in the present paper, direct evidence in support of this hypothesis was lacking, although data existed in the literature showing that simultaneous administration of androgen and oestrogen caused synergistic and antagonistic reactions, depending upon the proportions in which the hormones were supplied. The literature on this subject has been well reviewed by Burrows (1945) and will not be detailed here.

However, mention may be made of the following results. David, Freud and de Jongh (1934) demonstrated that the metaplasia and cornification of the prostate gland of the castrate male mouse produced by injection of oestrin could be prevented by the simultaneous injection of androgen. Laquer (1936) reported that injections of 10 μg. oestrone daily for 14 days produced metaplastic changes in the *uterus masculinus* of castrate male guinea-pigs. These effects were allayed or entirely prevented when, in addition, 500 μg. of dehydroandrosterone were injected daily over the same period.

[1] This work was carried out with the aid of a grant from the Wool Research Trust Fund.

This paper records the results of experiments designed to test the effect of regular administration of androgen to castrate male guinea-pigs fed on "potent" subterranean clover and to wethers depastured on "potent" subterranean clover.

EXPERIMENTS WITH GUINEA-PIGS.

Experimental Procedure.

Twenty-four male guinea-pigs were castrated under light ether anaesthesia and, after an interval of fourteen days, weighed and allotted to four groups of six animals each, so that the average weight of each group was approximately the same. A further group of six entire males was maintained as controls.

The animals were placed in cages in pairs and the groups treated for a period of eight weeks as follows:

Group I. Castrate males fed on 30 gm. dehydrated potent subterranean clover plus 15 gm. supplementary ration[2] daily.

Group II. Castrate males fed on a similar diet to Group I together with intramuscular injections of 1 mg. testosterone dipropionate in 0·1 ml. of peanut oil three times weekly.

Group III. Castrate males fed on 300 gm. fresh green grass plus 15 gm. of the supplementary ration[2] daily together with intramuscular injections of 5 μg. of stilboestrol in 0·1 ml. peanut oil every second day.

Group IV. Castrate males. Fed on "normal" diet similar to Group III but received no stilboestrol.

Group V. Entire males fed on same ration as Groups III and IV—no hormone treatment.

In addition, each animal in each group was given 70 mg. ascorbic acid at weekly intervals throughout the experimental period.

The clover which was very well consumed (26–29 gm./animal/day) was obtained from two sources in the spring when green and well-grown. It was dried for about 24 hours in an air-stream between 100° and 130° F. and stored in air-tight tins. Its oestrogenic potency was measured by the mouse injection technique developed by Robinson (1948). The first sample, taken from the grounds of the Institute of Agriculture, was found to have a potency equivalent to 3 μg. of oestradiol and the second, from property K in the "affected" area, a potency equivalent to 5 μg. of oestradiol. The first sample was fed to the guinea-pigs for six of the eight weeks and the second sample for the remainder of the period.

The animals of Group I therefore received a total of 52 μg. and of Group II 55 μg. of oestradiol equivalent over the eight weeks' period. Over the same period the animals of Group III received injections of stilboestrol totalling 120 μg. A direct comparison of oestradiol and stilboestrol, using the mouse injection technique employed for the clover samples, showed these two hormones to have very similar potencies. The stilboestrol-injected animals in Group III were thus receiving rather more than twice the oestrogenic potency of those in Groups I and II. The ratio of androgen to oestrogen in Group II was 440 testosterone: 1 oestradiol.

The body weights of all animals were obtained at weekly intervals; the nipple lengths at the beginning and end of the experimental period; the red-cell counts just prior to slaughter, and the individual weights of the accessory sex glands at slaughter. A histological examination of the secondary sex glands of all animals was also made. For this latter purpose the

[2] The supplementary ration consisted of bran 45 p.c., rolled oats 25 p.c., and dried skim milk 30 p.c. and was extremely effective in overcoming the marked toxic effects of a whole clover diet and in improving appetite and weight increases.

animals were killed at the end of the eight weeks' period and their reproductive organs removed, transferred to Vandergrift's (1942) fixative, embedded in paraffin and appropriate sections cut.

RESULTS.

The effects of the various treatments on body weights and red-cell counts will not be presented since the results were very variable, and this and certain other aspects are under further investigation with larger and more uniform groups. However, marked differences in the histological appearance of the structures examined and significant effects of the treatments on nipple lengths and on the weights of the secondary sex organs were obtained.

(a) *Organ Weights and Nipple Length.*

The mean nipple length and the mean weights of the secondary sex glands for the five groups at the end of the experimental period are presented in Table 1.

TABLE 1.

Effects of treatment on nipple length and weights of accessory sex glands of male guinea-pigs.

Group	No. in group	Treatment	Nipple length	Seminal vesicles	Bulbo urethral glands†	Coagulating glands	Prostate gland
			mm.	mg.	mg.	mg.	mg.
I	6	Castrated—clover diet	4·5	403	169	136	633
II	6	Castrated—clover diet + testosterone	3·0	1,261	225	210	867
III	6	Castrated—normal diet + stilboestrol	3·9	380	161	107	401
IV	6	Castrated—normal diet only	1·1	259	42	45	196
V	3	Entire—normal diet only	1·0*	1,070	223	193	819

* Mean of six (6) animals. † Plus 1 cm. of urethra.

Statistical examination of the nipple length results showed that the differences between Groups I and II and Groups II and III were significant at the 1 p.c. level. The slight difference between Groups I and III was not significant, indicating that the apparently small amount of oestrogen ingested in the clover had produced an effect quantitatively similar to that produced by the injected stilboestrol supplying twice the oestrogenic potency. The testosterone supplied to the animals of Group II, although effective, was not sufficient to protect entirely against the oestrogenic effects of the clover and maintain the nipples in the rudimentary state shown by the guinea-pigs in Groups IV and V. Macroscopic examination revealed that the nipples of Groups I and III were swollen, with, in most cases, the surrounding area darkly pigmented. The nipples of Group II were also somewhat turgid but pigmentation was not apparant.

Inspection of the data for the mean weights of the various accessory sex glands indicates that there were definite treatment effects. The atrophy due to castration was most evident, the values for Group IV being in all cases much lower than for any other group. There is also an obvious similarity between the organ weights of Groups I and III and between Groups II and V, indicating that the testosterone given to Group II had increased the weights of the secondary sex organs to approximately the levels of normal, entire animals. Similarly, there were no highly significant differences between Groups I and III, indicating that the oestrogen from the clover and injected stilboestrol had produced quantitatively similar effects, as in the case of nipple lengths. The position for Groups I and II differs somewhat for the different glands. The addition of the testosterone to the clover diet of the Group II guinea-pigs resulted in a highly significant (1 p.c. level) increase in the weights of their seminal vesicles, compared with those of Group I and a significant (5 p.c. level) increase in the weights of the prostates and coagulating glands. The difference in the mean weights of the bulbo-urethral glands in these two groups was not significant.

(b) *Histological Appearance of the Secondary Sex Organs.*

Microscopic examination made of sections of the seminal vesicles, vasa deferentia, urethra, coagulating glands, prostate gland and bulbo-urethral glands revealed striking and consistent differences in their histological appearance, in accordance with the treatment which the animals received (see Figs. 1–4). All these organs from the animals of Groups I and III showed changes identical with those reported by Burrows (1935) to be indicative of oestrogenic activity. Hyperplasia, characterized by intense and irregular proliferation of the lining epithelium of the organs, was followed by squamous metaplasia. In some cases keratinization and desquamation occurred. The extent and frequency of the keratinization was greater in the animals receiving the stilboestrol than in those consuming oestrogen from the clover in all the organs examined except the urethra, on which the oestrogenic effects were equally evident in both groups. In addition to these epithelial changes there was also an increase, sometimes very great, in the fibrous stroma of all organs.

Members of the second group, receiving testosterone in addition to clover, presented an entirely different picture. In these animals all the organs presented a development similar to the normal "entire" condition shown by the animals of Group V. Some scattered metaplastic tissues were evident, however, particularly in the epithelium of the ducts, and a slight fibrosis could be detected in most of the organs, these remnants being apparently indicative of a residuum of oestrogenic activity. The tissues of the castrate guinea-pigs comprising Group IV were in every case atrophic, as compared with the normal histological picture presented by the entire control Group V.

Since all the secondary sex organs examined in Groups I and III showed a considerable amount of hyperplasia or metaplasia, or both, it was difficult to determine from the results of

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EXPLANATION OF PLATE.

Fig. 1. Effect of subterranean clover diet on the prostate gland of the castrate male guinea-pig. Section of one prostatic tubule showing a sequence of epithelial changes. × 190. Note hyperplasia and dimensions of the fibrous stroma.

Fig. 2. Effect of subterranean clover diet on the prostate gland of a castrate male guinea-pig injected with androgen. Section of one prostatic tubule showing simple nature of the lining epithelium. × 300.

Fig. 3. Effect of subterranean clover diet on the bulbo-urethral gland of the castrate male guinea-pig. Section of the bulbo-urethral gland. × 190.

Fig. 4. Effect of subterranean clover diet on the bulbo-urethral gland of a castrate male guinea-pig injected with androgen. Section of the bulbo-urethral gland showing normal condition of the epithelium. × 300.

Fig. 1.

Fig. 2.

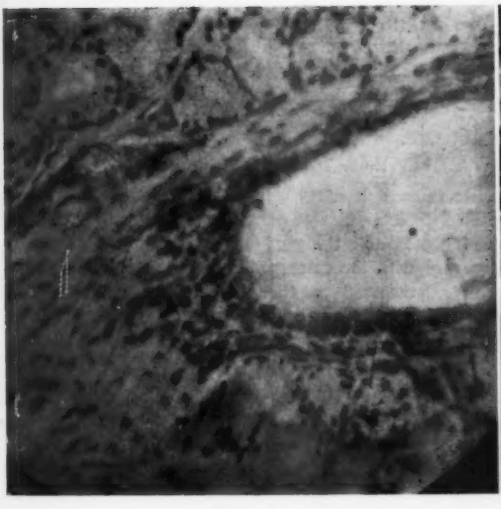

Fig. 3.

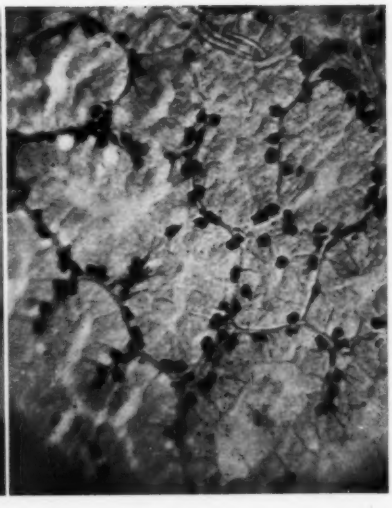

Fig. 4.

this experiment any differences in degree of responsiveness to oestrogen by the various organs. Gradients of responsiveness, however, were definitely shown within the seminal vesicles and bulbo-urethral glands. The epithelial changes in these organs were more marked in the ducts than in the acini.

EXPERIMENTS WITH SHEEP.

Experimental Procedure.

Twelve mature merino wethers from a property free from subterranean clover, were randomized into two groups of six and grazed together for nine weeks (July 15–September 16, 1947) on a pasture dominated by subterranean clover (*T. subterraneum* L. var. Dwalganup). Marked metaplastic changes had been found in the secondary sex organs of wethers grazed on this pasture for 13 weeks at the same period of the year in 1946 (Bennetts, 1947).

The conditions for both groups were identical except that in the test group each animal received an intramuscular injection of 20 mg. testosterone propionate[3] in 1 ml. of peanut oil three times a week throughout the experimental period. In the absence of any information on the amounts of androgen required to maintain normal sexual function in the sheep, the dosage was based on the approximate amounts required for this purpose in man.

Clinical observations were made on several occasions. On September 16 all the animals were slaughtered and the complete uro-genital tract was removed for examination. This comprised a macroscopic inspection of the organs concerned followed by a detailed examination of the bulbo-urethral glands and urethra. The bulbo-urethral glands plus a section of urethra from the caudal extremity of the glands to a point 4 cm. cranially were removed, weighed and then the whole fixed for 24 hours in 10 p.c. formalin. Following this primary fixation and hardening, a sagittal section was made through the middle region of the glands and attached urethra. After visual examination this section was transferred to Vandergrift's (1942) fixative and subsequently embedded in paraffin and prepared for histological examination. The inclusion of the portion of urethra was necessary for a comparison of weights of the glands because of the difficulty in enucleating highly metaplastic glands. A length of 4 cm. was chosen because it represented the outside dimension previously noted for a grossly affected gland, other than in the "cul-de-sac" formation described in earlier publications.

RESULTS.

The only notable clinical finding was an observation on September 9 that one of the androgen-treated wethers was attempting to serve ewes depastured in the same paddock.

Macroscopic examination of the uro-genital tracts indicated that the sex organs, notably the ampulla, seminal vesicles, bulbo-urethral glands and the penis of the androgen-treated animals coincided in size and general appearance with the corresponding organs of the ram, rather than with the smaller atrophic organs of the wether. Similar development in these organs was not seen in the untreated group and the bulbo-urethral glands of these animals showed macroscopic evidence of gross abnormality. The weights of the bulbo-urethral glands plus 4 cm. of urethra were as follows:

Clover grazing only	Mean 8·5 gm. (range 5·5–13·0 gm.)
Clover grazing + testosterone	Mean 8·5 gm. (range 7·2–10·0 gm.)
Non-clover grazing—normal wethers	Mean 4·3 gm. (range 3·6–5·1 gm.)

The differences in histological appearances in the two groups were very striking. In the treated group the appearance of the bulbo-urethral glands was constantly that of the normal functioning gland of the entire male. There was no evidence in any animal of hyperplastic

[3] The preparation used was "neohombreol".

or metaplastic process affecting any part of these glands or of the glands of Littre or of the portion of urethra included in the sections.

In the untreated group, however, marked changes attributable to the oestrogenic stimulus of the clover were present in all animals. The changes noted were those described previously, namely hyperplasia and squamous metaplasia affecting the bulbo-urethral glands, the glands of Littre and the epithelium of the urethra. The former glands in almost all instances showed the presence of numerous small cysts which are frequently found in the early stages of reaction, and have been attributed by Bennetts (1947) to an initial stimulation to secretion with a blockage of ducts which are affected with the metaplastic process earlier than the glandular acini.

GENERAL DISCUSSION.

The primary object of the experiments just described was to determine if the oestrogenic effects of "potent" subterranean clover on the secondary sex glands of the castrate male sheep and guinea-pig could be prevented by simultaneous injection of androgen. There was already evidence from the literature, as mentioned in the introduction, that androgens could "protect" various species against the effects of normal oestrogens.

The results of our experiments show clearly that the oestrogenic substance in subterranean clover, when ingested by castrate male guinea-pigs and sheep, is similarly affected by simultaneous injection of testosterone, at least in its effects on the secondary sex glands. In the case of the wethers, injection of 20 mg. of testosterone propionate three times weekly for nine weeks gave complete protection against the oestrogenic effect of pasture sufficiently potent to produce marked pathological changes, characteristic of oestrogens, in untreated animals in the same period. At this level of androgen administration these organs were not only protected against the ingested oestrogen, but showed a development in size and structure similar to that of the entire animal.

The experiments with guinea-pigs showed an essentially similar picture. At the levels of androgen given (1 mg. testosterone dipropionate on alternate days for eight weeks), a high degree of protection was afforded, but complete protection was not obtained. A small amount of scattered hyperplasia and metaplasia was evident, particularly in the ducts of the glands and some fibrosis could be detected in most of the organs of the androgen-treated animals. This suggests that the androgen level was not high enough in these animals, yet it was sufficiently high to induce a development in weight and structure of all the secondary sex glands similar to that found in a comparable group of entire males on a normal diet.

The results of the various treatments on the growth of the nipples in the guinea-pig are of interest, particularly when compared with those on the male accessory sex glands. The androgen exerted only a slight, although significant, protective effect on the nipples. The nipple lengths of the animals receiving oestrogen, either from the clover or from stilboestrol, with or without androgen,

were very much greater than in the case of the untreated entire or castrate males.

In general, the effects of oestrogen and of androgen were very similar in the two species studied. An exception was provided, however, by the seminal vesicles. In the guinea-pig this organ was found to be as susceptible to the oestrogen of the clover or to stilboestrol as the other organs examined. In the wether, on the other hand, Bennetts (1946) found only slight changes in the terminal parts of the ducts.

A point of some importance, which warrants further discussion, concerns the relative effects of the oestrogen ingested from the clover and the injected stilboestrol. The differences were actually very slight. In fact the results of the nipple length and organ weight measurements disclosed no significant differences due to treatment. The histological examinations do not lend themselves to statistical treatment, but the only difference which was at all consistent was a slightly greater degree of keratinization in the stilboestrol-treated, than in the clover fed, guinea-pigs.

Since the stilboestrol group was apparently receiving more than twice the oestrogenic potency of the clover group, the small degree of difference calls for some explanation. The most obvious one is that the effects in each case were either maximal or approached the maximum, which would, of course, eliminate any chance of proper comparison. It is possible that, if the experimental period had been restricted to four or six weeks instead of eight, some differences might have been revealed. This aspect is receiving consideration at the present time. Also the oestrogenic potency of the clover, on which the above comparison of intake was based, may have been greatly under-estimated by the assay technique used. Moreover, this assay was conducted with mice and an appreciable species difference may exist. These and other possibilities are being investigated at present and further work on other organs, notably the adrenals and pituitary gland, is being undertaken. These will form the subject of future publications.

SUMMARY.

Experiments were carried out to determine the effects of regular administration of androgen on the secondary sex organs of castrate male guinea-pigs and sheep (wethers) consuming diets composed largely of oestrogenically potent subterranean clover.

Intramuscular injection of testosterone propionate, three times weekly for nine weeks, completely prevented the development of any oestrogenic effects in the wethers, although untreated animals, under the same grazing conditions for the same period, showed marked changes attributable to the stimulus of the clover.

A high degree of protection, not quite complete, was afforded to guinea-pigs by similar injections of 1 mg. testosterone on alternate days for eight weeks.

The action of the androgen was much less marked in protecting against growth of the nipples than against changes in the male secondary sex organs.

In both species the androgen treatment resulted in the production of secondary sex organs comparable in size and structure to those of the normal entire male.

No significant differences in tissue reactions were observed between guinea-pigs consuming the potent clover and similar animals consuming a normal diet and receiving more than twice the total oestrogen in the form of stilboestrol.

The seminal vesicles of the guinea-pig showed typical oestrogenic effects from the clover diet. In the wethers slight changes in the terminal parts of the ducts of these organs only were observed.

Acknowledgments. Grateful acknowledgment is made to all members of the "team" of workers engaged upon the sheep infertility investigations for their help and advice and particularly to Mr. R. C. Rossiter of C.S.I.R. Division of Plant Industry for his assistance in the statistical analysis of results and to Mr. J. E. Peterson of the W.A. Department of Agriculture for supplying the figures for the weights of the bulbo-urethral glands from normal wethers.

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THE ACTION OF YOHIMBINE ON EXCITATION AND PROPAGATION IN NERVE

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In the past a considerable amount of work has been done on the action of such drugs as alcohol, cocaine, veratrine and eserine in various phases of nervous activity, but little has been done with other drugs. It is our intention to investigate the action of other drugs which influence nervous activity and for the first we have chosen yohimbine, as we have had in the past considerable experience with this substance (Shaw, 1948).

The previous references to the use of yohimbine in this connection are those of Tait (1910), who found a remarkable lengthening of the relative refractory period and a less marked effect on the absolute refractory period, and Graham (1935) who worked almost exclusively on the measurement, by means of the cathode ray oscillograph, of the excitability during "after action" phases.

EXPERIMENTAL.

The preparation used was the gastrocnemius of the toad, *Bufo marinus*. In the beginning diphasic action currents were registered because we also wished to observe the muscular contraction. As the work progressed it was observed that the degree of contraction of the muscle paralleled the height of the action current and we then changed over to monophasic responses, which were obtained by crushing the nerve and dipping the crushed portion in 1 p.c. potassium chloride. The concentration of yohimbine hydrochloride employed varied from 1 in 1,000 to 1 in 3,000. This is about the limit of solubility of yohimbine in Ringer's solution and it was often necessary to warm the liquid to about 40° C. to obtain complete solution. Care was always taken to do this gradually to prevent precipitation of the calcium. The preparation was mounted in a paraffin chamber with a glass lid. A bath containing the yohimbine was raised or lowered through the floor of this chamber and the portion of nerve which we desired to treat could then be immersed in this bath. The electrodes which served the middle portion of the nerve (see Figs. 1, 2) were movable and before any readings on the oscilloscope were made the bath and all idle electrodes were removed.

In all cases the stimulus was adjusted to just threshold for maximal response from the A fibres.

The electronic apparatus consisted of stimulator, pre-amplifier, oscilloscope and special time base. The stimulator was of the hard valve type, with a triggered multivibrator circuit to give a variable square wave duration, diode limiters to control amplitude and a second

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council.

multivibrator to trigger the first for repetitive stimulation. The output was a cathode follower to ensure a low impedance source of stimulus to the tissue, with provision to balance out all direct current in the output.

The preamplifier contained a two stage, direct coupled, push-pull pentode circuit with a high resistance common to the cathodes of each stage, and running to a negative source. This gave a good discrimination against in-phase potentials, and helped to minimize stimulus artefact. The final amplifier was condenser coupled to the preamplifier and to the Y plates of the cathode ray tube.

The time base was designed to trigger from the stimulator pulse. In the stable condition of the time base the charging condenser was in the discharged state. The pulse triggered a

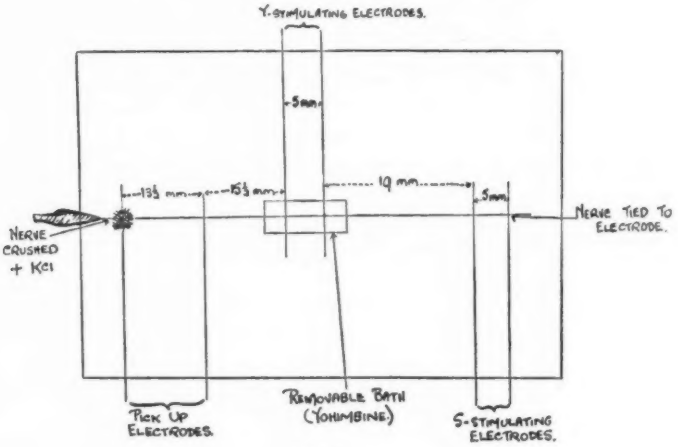

Fig. 1

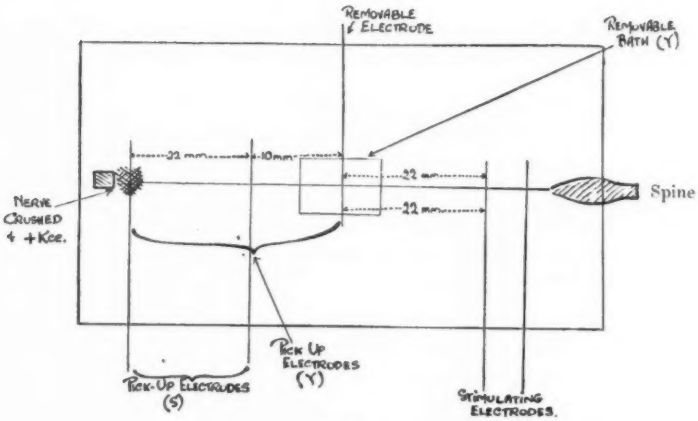

Fig. 2

Schmitt circuit which cut off a triode in parallel with the charging condenser, and synchronously delivered a positive square wave pulse to the grid of the cathode ray tube which had been biased to extinguish the beam. This brought up the beam intensity at the same time as the beam was deflected by the charging of the condenser. At the completion of the spot traverse, the circuit reverted to its stable condition with extinction of the spot and return to its initial position. This circuit will be fully described in a future paper.

The electrodes were either platinum or silver wire.

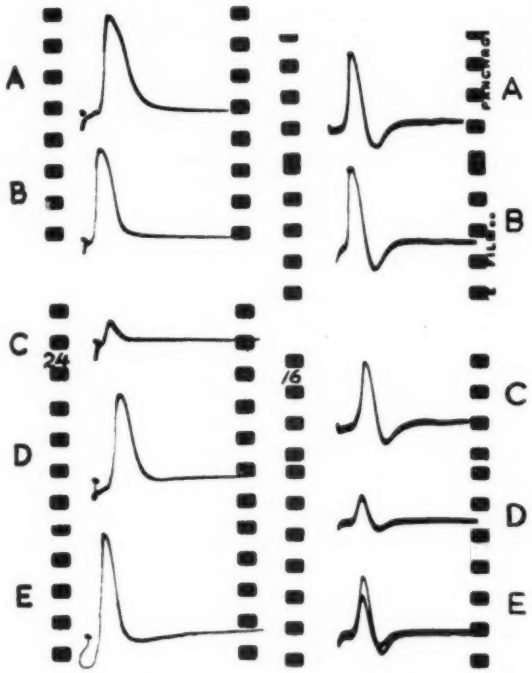

Fig. 3.

Fig. 4.

Fig. 3. Showing transmission of impulse through a yohimbinized region when the region does not respond to a stimulus previously sufficient to excite all the A fibres, but which does respond if the stimulus is increased.

A. Spike from peripheral portion when spinal end stimulated (0·5 volts, 1·2 m.secs.).

B. Spike from peripheral portion when middle region stimulated as in A. 45 minutes' interval, during which middle region has been treated with yohimbine.

C. As in A. D. As in B. E. As in D, but stimulus now 2·0 m.secs.

(For time and voltage calibration, for this and all other Figures, see Fig. 5.)

Fig. 4. As for Fig. 3 (diphasic response).

E. Smaller trace stimulus time 1·8 m.secs. larger trace stimulus time 2·2 m.secs.

1. *Threshold and Velocity Changes in a Treated Region.*

The position of the electrodes is set out in Fig. 1.

From this it will be seen that the nerve was stimulated both before the treated region and in the treated region and that the impulse was picked up in the peripheral untreated area.

The results showed that during the first two hours, depending upon the individual variation of the nerve, the threshold in the untreated was unaffected and that the impulse was conducted without loss through the treated region. On the other hand, after a half to one hour it was necessary to raise the strength of the stimulus in the treated region to obtain a spike of height comparable with that obtained from the untreated portion (see Figs. 3, 4). Strength of stimulus was measured either by voltage or duration. In our experiments the average stimulation was one of 0·5 volts acting for 0·1 m.secs. In the yohimbinized area this had to be increased gradually to 0·7 volts or 0·25 m.secs. (one or the other being held constant). In a few of

the experiments there was a delayed rise of the threshold in the untreated portion of the nerve and if the stimulus were increased a larger spike was obtained from the untreated region. This would make it appear that the size of stimulus affected the conduction through the depressed area. This was not so. These few aberrant results were due to diffusion of yohimbine from the bath to the electrodes in the untreated portion and when the distance of the bath from the electrodes was maintained at 15 mm. this phenomenon did not occur. There was also evidence that the threshold of the fastest fibres was affected before that of the slower fibres because, as the excitability of the nerve decreased, the crest of the wave moved to the right and at the same time the amplitude lessened. If the strength of stimulus was now raised the amplitude was restored and the peak of the spike moved to the left.

From these results it was concluded that, whilst excitability is impaired in the yohimbinized region, conduction is unaffected. It might be argued that increasing the strength of stimulus in the treated region resulted in stimulation of new fibres below the treated region by spread of current (cf. Davis, 1926). This objection was answered by the fact that at the raised threshold, fatigue (Section 2) was still obtained. This would not have been so if fibres in an untreated region were being stimulated. This fact was confirmed by placing electrodes below the bath and passing in repetitive impulses when fatigue was not obtained.

As the time of treatment progressed the time at which the impulse reached the pick-up electrodes lengthened. The velocity of the impulse which at the commencement of the experiment was usually in the vicinity of 33 metres per second fell to about 17 metres per second after about two to three hours. About the same time or shortly after the decrease in velocity was evident the height of the spike began to diminish, as though certain more susceptible fibres were being blocked (see Section 3).

A further consideration of alteration of velocity is deferred until the results with repetitive stimulation (fatigue) are given (Section 2).

The action of yohimbine was reversible but not readily so, about 4 hours' soaking in fresh Ringer's being required before recovery of threshold or velocity was complete.

Another point of perhaps more importance was the fact that the conduction rate may be slowed as much as 25 p.c. before there is any reduction in the height or area of the action current. This was not a constant finding, but it was evident in a few of our experiments. However, the results did indicate that a decrease in velocity could occur without decrease in amplitude but that in most experiments fibres began to be blocked before this slowing was evident (Fig. 7).

2. *The Fatigue Phenomenon.*

If the stimulating electrodes were placed at the spine end of the preparation, the pick-up electrodes in the yohimbinized area, and at the muscle end of the preparation, and repetitive shocks sent in, the following phenomena were observed. (For position of electrodes see Fig. 2.)

After about half an hour, successive stimuli of a repetitive burst caused increasing reductions in the action potentials developed in both treated and normal areas. The effect which was first obtained with stimuli at the rate of about 50 per second resulted in a reduction of the height of the action current to about 40 p.c. of its initial value after about a second (Fig. 5). As time progressed, the fatigue phenomenon became more marked and after about 1–2 hours' stimulation at the rate of 4 per second produced complete cessation of conduction after about 5 shocks. If stimulation was discontinued the nerve recovered in from 1–10 seconds and the phenomenon could be repeated as often as was desired. From these results it can be said that the absolute recovery (refractory) period was increased by yohimbine to more than 0·25 seconds (and in several cases it was actually extended to 1 second) and the relative recovery period (time to complete recovery after fatigue) to about 5 seconds, with an extreme value of 15 seconds.

The fatigue effect seems to be specific for yohimbine (as far as has been tested) for it is not obtained with alcohol, eserine or various barbiturates (veronal, luminal, nembutal).

During repetitive stimulation, in addition to fatigue effects, changes in velocity were also noticed. As has been mentioned in the previous section, the velocity of the wave in the yohimbinized area was decreased and if repetitive stimuli were thrown in there was a further decrease in velocity. This was concluded from the following: the commencement of the wave was later, the peak had moved to the right and finally the wave ended later. Concomitant with this shift of the wave to the right there was, of course, a diminution in height. It is obvious that the fastest fibres were fatiguing before the slower ones and it might be argued that there was not a real change in velocity but only an apparent one. However, the fact that the wave also ended after that of a single stimulation means that some fibres at least had a velocity less than before.

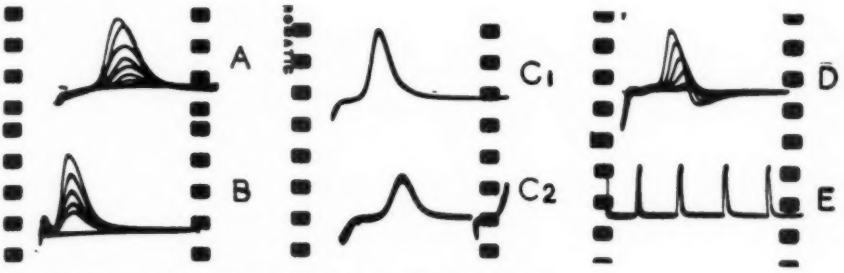

Fig. 5. Showing various phases of fatigue phenomenon.

A. Repetitive stimulation at 6 shocks per second. Note moving to the right of the crests showing slowing of velocity with fatigue.

B. Repetitive stimulation at 4 shocks per second. Note very little alteration in velocity.

C_1. Single shock. C_2. After 30 seconds at 10 shocks per second (nerve only treated 30 minutes). Note marked change in velocity.

D. Complete fatigue at 4 shocks per second. Diphasic response. Note the gradual change to monophasicity as fatigue progresses.

E. Calibration. Vertical, 10 m.volts., horizontal, each space 2 m.secs.

Fig. 5 gives an example of what has gone before and describes the results which are usually obtained. As is well-known, there is a tendency for the crushed end to "seal over" after a few hours. The response then tends to become diphasic and, of course, will alter the above picture. This danger has always been guarded against here and in other sections of this work. (The phenomenon of diphasic artefact was also encountered). Occasionally it has been observed that just before the nerve was almost fatigued, the now small spike was "inside" the original wave (Fig. 5). This probably means that there are some very resistant fibres of moderate velocity. What might be their anatomical or functional relationships is difficult to say, and the investigation has not been pursued further.

3. *Block or Decrement.*

The next point to be determined was whether the reduction brought about in potential by the yohimbine was a decrement which recovered when the impulse reached a fresh portion of nerve distal to the yohimbinized area or whether there was a progressive total blockage of fibres in the treated area. To test this the set up of Fig. 2 was employed.

The results indicated that as the potential fell off in the treated area it did not at any stage pick up again in the fresh nerve. That is to say, the conduction in the yohimbinized area is truly all or none.

To ensure that the decrement had not been "missed" we repeated Davis and Forbes (1924) work with alcohol. In this instance, we did obtain a diminution in the height of the action current (varying from 5 to 20 p.c.) in the treated region, which picked up when the untreated nerve was reached.

4. *Change from Diphasicity to Monophasicity.*

When the electrodes were arranged as in Section 2 and a diphasic response was being recorded it was noticed that very often the second wave of the diphasic response became less and less until to all intents and purposes a monophasic response was obtained. This phenomenon was always present during repetitive stimulation.

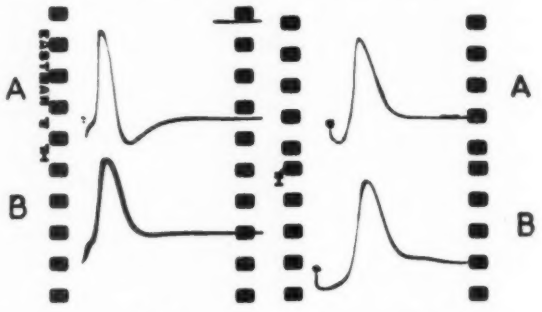

Fig. 6.

Fig. 7.

Fig. 6. Diphasic response becoming monophasic when stimulated at the rate of 20 per second 40 minutes after treatment with yohimbine.

Fig. 7. Showing alteration in velocity without alteration in amplitude.

A. Spike at 0 minutes.

B. Spike after 35 minutes treatment with yohimbine.

A preparation set up to register a diphasic wave would after the passage of a few impulses become monophasic before it eventually fatigued (see Fig. 6). These results recall those of Gerard (1932) and Bishop (1932).

Changes in negative after potentials were noticed but no regularity was perceived and a study of these and positive after potentials will form the subject of another publication.

DISCUSSION.

Several interesting points arise from the results which have been obtained by treating nerve with yohimbine and they will be dealt with in the same order as they have been treated in the foregoing sections. Firstly, yohimbine has been found to produce an elevation of the threshold for electrical stimulation. This, in itself, is not unusual, as many others drugs do the same (carbon dioxide, ether, chloroform and alcohol). However, our results show conclusively that, although excitation is depressed in the treated area, conductivity is normal. This is in agreement with the results of the earlier workers with alcohol, etc., and against the findings of Adrian and Lucas (for references, see Schafer, 1900, and Bayliss, 1924). It should be noted that the technique of the earlier workers and ourselves is similar in that it involved direct measurement of the action currents, whilst the conclusions of Adrian and Lucas are based on deductions from the

results of experiments which depended on an interpretation of summation curves for muscle contraction. We feel that our results show that with yohimbine it is possible to separate excitability from conduction in a stretch of nerve.

That the threshold for stimulation is raised before the velocity is altered calls for comment. According to Hermann (1879), theory requires that as the threshold is raised the velocity should be slowed. In all our experiments it has been possible to show that the threshold is raised within a few minutes of the application of the drug, but that the velocity does not fall until about one hour afterwards. Before the velocity alters it is found that the magnitude of the spike in the peripheral untreated region (measured as height or area) begins to decrease. This is interpreted to mean that some of the fibres are being blocked in the treated region. However, in a few experiments it was found that the velocity was slowed before the magnitude decreased (see Fig. 7). This result recalls the earlier work of Adrian from which he concluded that the velocity of conduction in narcotized nerve was independent of the magnitude of the impulse. However, Davis and others (1926) point out that the membrane theory demands a definite relationship, namely, that the velocity be proportional to the magnitude (and the rate of development of the electrical potential). This Forbes (1923) had shown to be the case when the impulse was travelling in the wake (relative refractory period) of another impulse. Our results agree with those of Adrian in so far as the magnitude is independent of the velocity in a few of our experiments.

With regard to the fatigue phenomenon, we use the term "fatigue" loosely and perhaps a stricter expression would be to say that the absolute recovery (refractory) period, after several stimuli, has been lengthened from the usual value of 1·5 m.secs. to about 250 m.secs. The only comparable phenomenon is "Wedensky Inhibition". This term has been used widely for a variety of phenomena concerned with transmission in the nerve and at the neuromuscular junction, and even for propagation in the muscle itself. It is our intention to restrict the term as nearly as possible to the phenomenon as described by Wedensky and other early workers (Bethe, 1929). The phenomenon is twofold. Firstly, repetitive stimulation of the fatigued or narcotized nerve at high intensity is without effect (except for the first impulse), but stimulation at the same rate at low intensity will send the muscle into tetanus. This is the condition dealt with by Lucas and which he says is due to the ability of a strong stimulus in the relative refractory phase to produce further local excitation and so lengthen the refractory period indefinitely, whereas a weak stimulus is unable to do this and certain of the stimuli pass through at the end of each refractory period and so the muscle receives sufficient stimuli to produce a tetanus. Both Adrian and Lucas are quite definite that alcohol does not lengthen the refractory period and so this explanation of the inhibition is not possible. Another way of demonstrating the Wedensky Inhibition is to show that, to a narcotized

nerve, stimulation at a rate greater than 30 per second results in an initial contraction followed by inhibition, whilst stimulation at a rate less than this would result in a tetanus.

We do not think our results are comparable with those of Wedensky Inhibition. In the first place, we obtained inhibition with impulses at the rate of 4 per second, far lower than that reported by other workers. Secondly, the inhibition comes on gradually, whereas the Wedensky Inhibition is heralded by an initial contraction, then silence. It would appear, in the case of yohimbine, that there is an interruption of normal metabolism. It is felt that the next step is a determination of the action of yohimbine on the various enzyme systems which may be concerned in the metabolism of nerve.

SUMMARY.

Yohimbine raises the threshold of nerve to electrical stimulation without at first affecting the conductivity.

The impulse is propagated more slowly in a yohimbinized nerve.

The absolute and relative recovery (refractory) periods are increased after repetitive stimulation and a state resembling fatigue is produced.

Yohimbine does not produce any decrement in the size of the action potential but can produce total block in a fibre.

Under certain conditions a diphasic response may become monophasic.

The importance of some of these observations as they apply to nerve physiology is discussed.

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